# 2
# Multivalency in Ligand Design

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## 2.1
## Introduction and Overview

We define *multivalency* to be the operation of multiple molecular recognition events of the same kind occurring simultaneously between two entities (molecules, molecular aggregates, viruses, cells, surfaces; Fig. 2.1). We include in this definition hetero-multivalency (i.e., interactions in which two or more *different* types of molecular recognition events occur between the two entities), but do not discuss this type of system in detail (representative examples are sketched in Section 2.7.1; Fig. 2.1 b). Hetero-multivalency is probably a more broadly applicable concept than homo-multivalency, but one whose underlying principles are the same. Homo-multivalency is, however, simpler to understand than hetero-multivalency. We elaborate on our definition of multivalency in Section 2.2.1.

Multivalency is a design principle that can convert inhibitors with low affinity ($K_d^{affinity}$ ~ mM – μM) to ones with high avidity ($K_d^{avidity}$ ~ nM) and/or biological "activity" (gauged by some relevant parameter: for example, values of $IC_{50}$; the concentration of free ligand, often approximated as the total ligand, that reduces the experimental signal to 50% of its initial value) [1]. We discuss the distinction between "affinity" and "avidity" in Section 2.2.1, but emphasize here that high

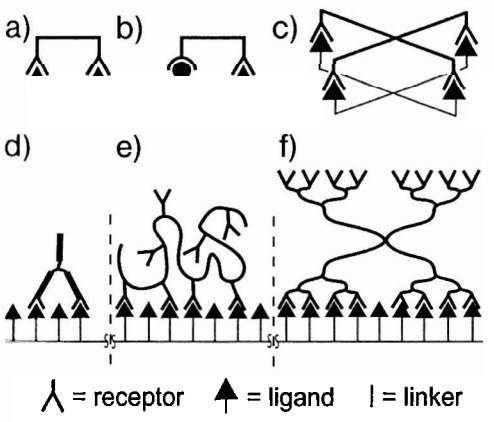


**Fig. 2.1**
Types of multivalent systems: (a) a bivalent ligand binding to a bivalent receptor ($N = M = i = 2$), (b) a heterobivalent ligand binding to a protein with two different kinds of binding sites ($N = M = i = 2$), (c) a tetravalent ligand binding to a tetravalent receptor (oligovalent) ($N = M = i = 4$), (d) a bivalent antibody binding to a surface ($N$ = unknown, $M = i = 2$), (e) a polymer binding to a surface ($N$ = unknown, $M = 8$, $i = 4$), and (f) a dendrimer binding to a surface ($N$ = unknown, $M = 16$, $i = 8$).

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avidity does not necessarily require high affinity. Multivalency provides a strategy for designing ligands against defined oligovalent systems containing multiple, identical binding sites (e.g., antibodies, complement, multi-subunit toxins, such as those with an $AB_5$ structure). In addition, multivalent approaches can be effective in generating high-avidity ligands for proteins with multiple binding sites from low-affinity ligands. (One or more of these binding sites can, in some instances, even have low structural specificity and still be effective in increasing avidity: a hydrophobic patch adjacent to the active site of a monovalent enzyme can, for example, serve as the second binding site in a hetero-bivalent interaction.)

Multivalent ligands (primarily polyvalent ones, see Section 2.2.1) are especially well suited for inhibiting or augmenting interactions at biological surfaces (e.g., surfaces of bacteria, viruses, cells; Fig. 2.1d–f): they can prevent adhesion of these surfaces to other surfaces [2, 3] (e.g., by grafting polymers to the surfaces of viruses to prevent adhesion to cells) [4–10], or cluster cell-surface receptors to induce downstream effects [11–13]. By using polymers that display multiple kinds of ligands as side-chains, multivalency can convert a surface having one set of properties into one with different properties [14, 15].

This chapter sketches a theoretical analysis of multivalent systems that is intended to guide the application of multivalency to the *design* of high-avidity ligands for appropriate biological targets. The chapter has seven parts: (i) introduce the nomenclature of multivalency and the qualitative concepts that characterize it, (ii) present key experimental studies to provide examples of particularly tight-binding (high-avidity) multivalent ligands, (iii) present theoretical models that describe multivalent systems, (iv) explore representative multivalent ligands in the context of these models, (v) provide design rules for multivalent ligands, (vi) discuss extensions of multivalency to lead discovery, and (vii) mention challenges and unsolved problems for the application of multivalency in ligand design.

## 2.2 Definitions of Terms

In this chapter, we focus on representative systems that exhibit the principles of multivalency, and that suggest approaches to new types of drug leads α ligands (i.e., the design of ligands to interact tightly with multivalent receptors – primarily proteins). There are several thorough reviews on both experimental [16–18] and theoretical [1, 19, 20] aspects of multivalency. The book by Choi is an excellent compilation of experimental results; it also discusses potential targets (e.g., receptors on pathogens, multivalent proteins, etc.) for multivalent ligands [16].

We use the terms "ligand" and "receptor" to identify the individual components of multivalent species: the *receptor* is the component that accepts the *ligand*, using a declivity or pocket on its surface (Fig. 2.1). We refer to the entire molecule or cluster of molecules that present the receptors as "oligovalent receptors" (with an analogous relationship between ligand and "oligovalent ligands"). The *linker* is the tether between ligands in the oligovalent ligand. While there is also a linker be-

tween receptors, we do not discuss this linker in this chapter because it is usually not subject to manipulation by the investigator (it is part of a naturally occurring structure).

We make a distinction between the total number of ligands (and receptors) in an oligovalent species and the number of interactions between an oligovalent ligand and receptor. We denote the total number of ligands (the valency of the oligovalent ligand) with $N$ and the total number of receptors with $M$. The number of receptor–ligand interactions between the two oligovalent species (in a particular state: Section 2.4.2) is $i$ (Fig. 2.1).

We divide multivalency, on the basis of the number of interactions ($i$) between the multivalent receptor and ligand, into three categories: (a) bivalency (Fig. 2.1a, d), with two interactions between the different species ($i = 2$; for example, IgG and IgE binding to a cell surface; Fig. 2.1d), (b) oligovalency (Fig. 2.1c), with a discrete number (which we arbitrarily define as $i \leq 10$, a number that includes the pentameric immunoglobulin, IgM [21], and some interactions involving pentameric toxins) of interactions, and (c) polyvalency (Fig. 2.1e, f; usually associated with polymers), with a large number ($i > 10$) of interactions between the two species (the exact number of which is often unknown). While bivalency and oligovalency are distinct in mechanism, the thermodynamics of the two are sufficiently similar that we discuss them together in the remainder of the chapter and refer to them, collectively, as oligovalency. Polyvalency differs fundamentally from oligovalency both in terms of thermodynamics and mechanism; and we discuss it separately.

There are several different thermodynamic terms in the literature that have been used to describe the binding strength of multivalent ligands to multivalent receptors. The *affinity* of a monovalent interaction is defined by its dissociation constant ($K_d^{affinity}$); and this constant usually has units of concentration (typically, molarity; Fig. 2.2a). We define the *avidity* ($K_d^{avidity}$) of a multivalent interaction to be the dissociation constant ($K_{d,N}$) of the completely associated receptor–ligand complex with $N$ receptor ligand–interactions ($i = N$) relative to the completely dissociated ($i = 0$) forms of the multivalent receptor and ligand, Eq. (1):

$$K_d^{avidity} \equiv K_{d,N} \tag{1}$$

An example with a bivalent ligand and receptor ($N = 2$) is shown in Fig. 2.2b. Kitov and Bundle [22] proposed an alternative definition of avidity ( ), given in Eq. (2):

$$K_d^{avidity,KB} \equiv (1/K_{d,N} + 1/K_{d,N-1} + \ldots + 1/K_{d,1})^{-1} \tag{2}$$

where $K_{d,N}$, $K_{d,N-1}$, and $K_{d,1}$ are the dissociation constants of receptor-ligand species with $N$ receptor–ligand interactions ($i = N$), $N-1$ interactions ($i = N-1$), and one interaction ($i = 1$), respectively, to the completely dissociated multivalent receptor and multivalent ligand ($i = 0$; Fig. 2.2).

This definition of avidity [Eq. (2)] is more general than that in Eq. (1) because it takes into account all of the receptor–ligand species in solution and will prove par-

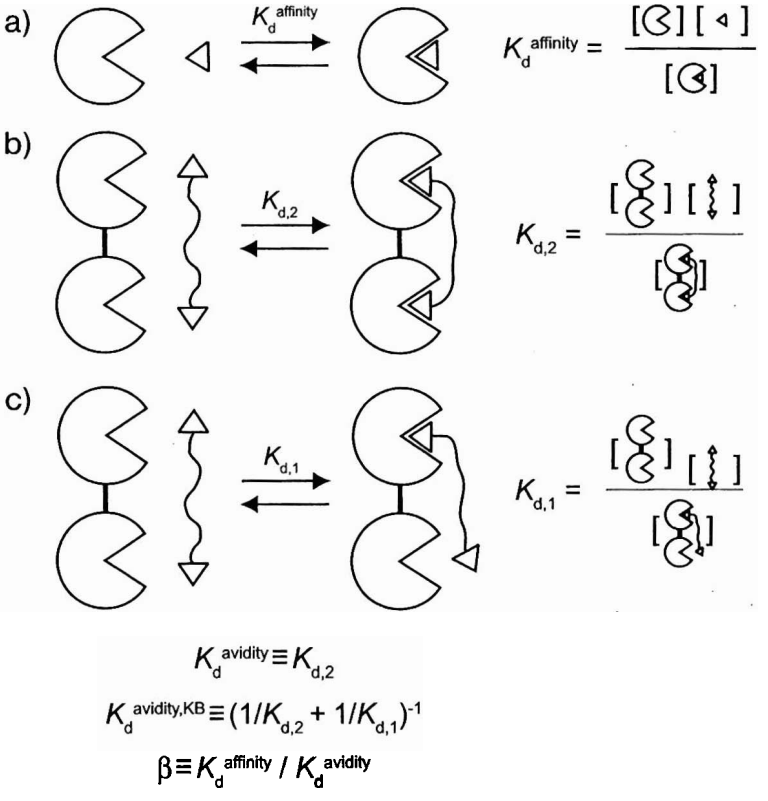



**Fig. 2.2**
Thermodynamic equilibria used for the definitions of affinity, avidity, and enhancement. (**a**) A monovalent ligand binds a monovalent receptor with a dissociation constant of $K_d^{affinity}$. (**b**) The oligovalent ligand (here, bivalent) binds a receptor of the same valency with a dissociation constant of $K_{d,2}$ for the equilibrium between the fully complexed receptor and free receptor and ligand. (**c**) The bivalent receptor can also bind the bivalent ligand with only one receptor–ligand interaction; the complex has a dissociation constant of $K_{d,1}$. We define the avidity ($K_d^{avidity}$) as $K_{d,2}$ (the equilibrium in b). Kitov and Bundle [22] defined the avidity ($K_d^{avidity,KB}$) to take into account all receptor-ligand complexes in solution. The enhancement ($\beta$) is the ratio of the affinity to the avidity. In this case, the enhancement contains a contribution from a statistical factor of 2.

ticularly useful when the binding of even one receptor of a multivalent receptor by a multivalent ligand is enough to achieve the desired response (e.g., in certain cases of inhibition of binding to a surface). Application of Eq. (2) is less convenient than of Eq. (1), because the distribution of receptor–ligand complexes must often be modeled and often cannot be measured directly. This difficulty prevents a simple analysis of the separated thermodynamic parameters of enthalpy and entropy (see Section 2.4.2) that is the focus of the approach that we present here. Further, the definition that we propose [Eq. (1)] will be more relevant than Eq. (2) for multivalent systems in which all of the receptors of the multivalent receptor must be bound to achieve the desired biological response (e.g., inhibiting multiple catalytic sites of a multimeric enzyme). Finally, in the design of ligands that are to serve as the starting points for possible drugs to bind oligovalent receptors with fewer than three receptors, the assumption that the fully associated complex

($i = N$) is the predominant receptor–ligand complex is justifiable because, in many of these cases, the multiple receptors are in close proximity to one another. We, thus, only discuss the avidity as defined by Eq. (2) in depth, but provide a general model for the free energy of binding of all species in solution in Section 2.4.2 before restricting our analysis to the fully associated state ($i = N$).

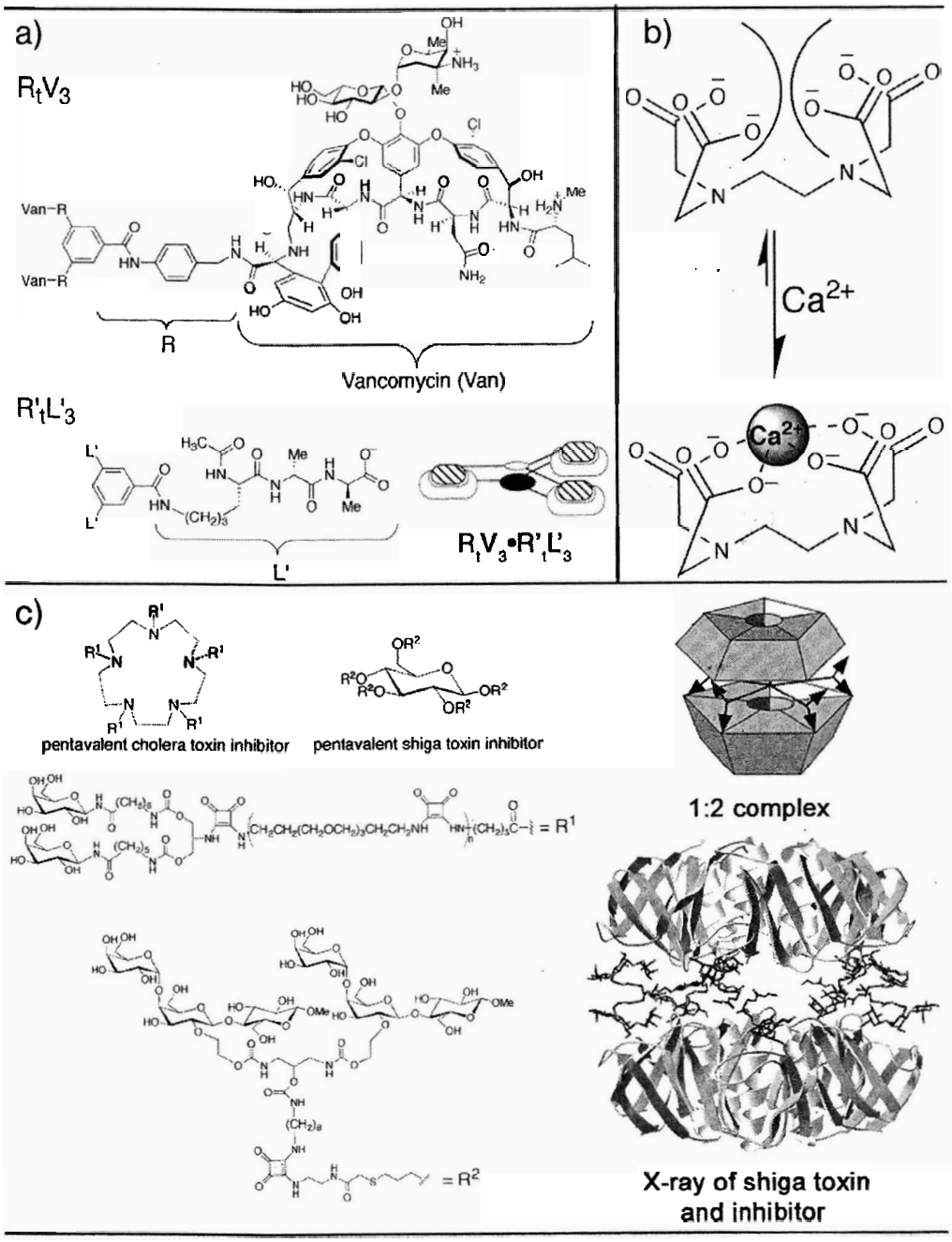
We previously defined the enhancement (β) due to multivalency as the ratio of $K_d^{avidity}$ to $K_d^{affinity}$ (Fig. 2.2); this parameter gives a measure of the benefit of having several ligands linked together [1]. This definition of enhancement does not take statistical factors (or influences of the topology of the ligand; Section 2.4.2) into account; and so the enhancement will increase with valency on a statistical basis alone (Fig. 2.2). This definition, however, enables the use of a general, empirical parameter for describing multivalency in many systems (and in those systems with an unknown number of interactions between species).

For polyvalent ligands (e.g., polymers), values of avidity and enhancement can be defined as above: $K_d^{avidity}$ would be the concentration of free polyvalent ligand that results in a measured signal of one-half of the maximum signal [22]. An enhancement calculated on this basis would have a significant contribution from statistical effects due to the large number of ligands (even without any "real" benefit of linking the ligands together). A theoretical understanding of the enhancement (Section 2.4.2) is complicated, because the number of ligands directly binding to receptors is sometimes unknown, and often known only approximately [4–8, 10]. The attachment of a polyvalent ligand to a surface, for example, may be very effective in changing the properties of that surface, even if only a few of the receptors on the surface are occupied [1, 4, 15] Another approach to defining the avidity of a polyvalent ligand is to calculate $K_d^{avidity}$ on a "per ligand" basis. An enhancement calculated using the "corrected" $K_d^{avidity}$ gives a qualitative correction for statistical factors, although again the unknown number of interacting ligands complicates rigorous analysis. Kitov and Bundle discussed the shortcomings of this approach based on "corrected" values of $K_d^{avidity}$ (Section 2.4.2), but there is no obviously better, general approach [22].

Frequently, estimates of avidity ($K_d^{avidity}$) are difficult to obtain experimentally and to interpret theoretically: polymers provide a prime example. Another term, related to avidity, but sometimes experimentally more tractable, is "biological activity" (sometimes measured, in familiar terms, as an $IC_{50}$; Section 2.5.1). Values of biological activity have the advantage that they are often measured under conditions that better simulate biological environments (e.g., at surfaces) than do the conditions usually used in the laboratory to measure values of $K_d^{avidity}$ (e.g., in solution). The two kinds of values (biological activities and $K_d^{avidity}$) are qualitatively similar (e.g., the qualitative ordering of ligands will usually be the same for both), but the exact values of biological activities are often assay-specific, while values of $K_d^{avidity}$ are less sensitive to the details of the assay. Kitov and Bundle have recently provided a theoretical framework for interpreting values of biological activities in certain contexts [22].

## 2.3
## Selection of Key Experimental Studies

We have selected five representative examples that highlight key theoretical aspects of multivalent systems (Fig. 2.3). All of these systems are studied in aqueous solutions. We have not considered examples in organic solvents [23–28]. These examples may illustrate general principles of multivalency, but have no direct relevance to biochemistry or biology occurring in aqueous solutions. We do not mean the examples discussed here to be comprehensive, but merely representative of



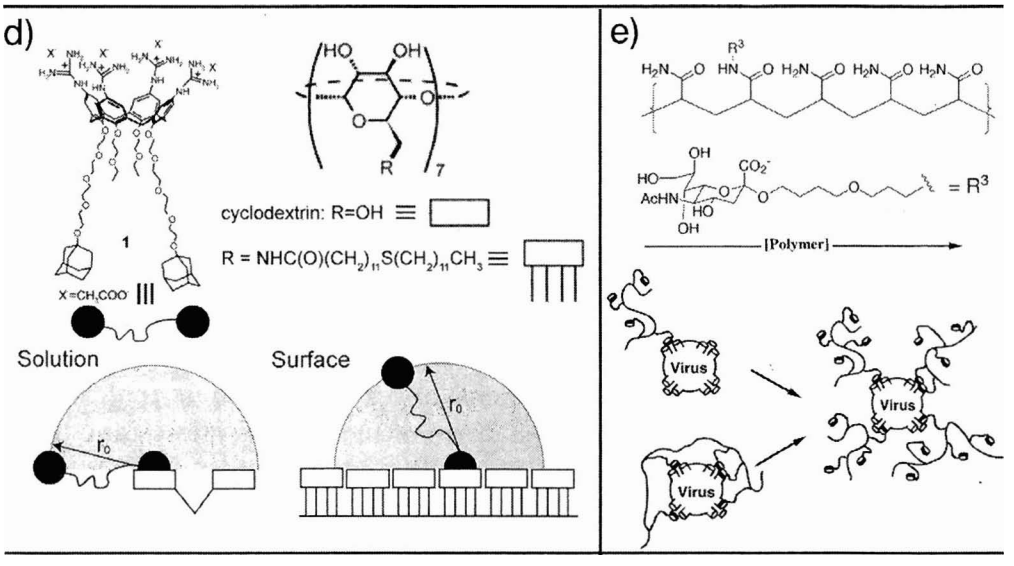



**Fig. 2.3**
Key experimental systems discussed in the text. (**a**) Trivalent Vancomycin-D-Ala-D-Ala [29, 30] (adapted with permission from [30]; copyright 2000, American Chemical Society). (b) EDTA/$Ca^{2+}$ [31]. (**c**) $AB_5$ toxin inhibitors: Shiga-like [32] and cholera [33] toxin inhibitors (adapted with permission from [33]; copyright 2002, American Chemical Society). The ribbon diagram was generated using Swiss PDB Viewer and atomic coordinates: PDB 1QNU [32]. (**d**) Bivalent adamantane binding to bivalent cyclodextrin in solution and to SAMs displaying cyclodextrins [34] (adapted with permission from [34]; copyright 2004, American Chemical Society). (**e**) Polymers (polyacrylamide) displaying sialic acid for binding flu [8] (adapted with permission from [8]; copyright 1996, American Chemical Society).

studies that illustrate elements of multivalency. In Section 2.7 we present further examples and applications of multivalency to lead discovery. Choi reviewed experimental examples of multivalency in a more comprehensive fashion than we do here [16].

### 2.3.1
### Trivalency in a Structurally Simple System

The trivalent vancomycin · D-Ala-D-Ala system is an example of the use of oligovalency to convert an interaction that is moderately strong for the monovalent species ($K_d^{affinity} \sim \mu M$) into one that is *very* strong – in fact the highest affinity known ($K_d^{avidity} \sim 10^{-17}$ M) for a ligand–receptor interaction involving species of low molecular weight (relative to those of proteins; Fig. 2.3a) [29, 30]. This system also illustrates the difference in mechanism (manifested in the kinetics) between tight-binding oligovalent systems and tight-binding monovalent ones: the trivalent vancomycin · D-Ala-D-Ala complex dissociates rapidly in the presence of competing monovalent ligand (equilibration of 3 μM of the trivalent complex vancomycin · D-Ala-D-Ala with 86 mM (~17 000 $K_d^{affinity}$) of diacetyl-L-Lys-D-Ala-D-Ala was complete in <45 min), while monovalent complexes of comparable affinity (e.g., biotin-avidin, $K_d^{affinity} \sim 10^{-15}$ M) dissociated slowly (half-life for dissociation

~ 200 days) [35]. This kinetic observation illustrates that the mechanism for dissociation (and association) of oligovalent species is qualitatively different than that for tight-binding monovalent species such as biotin–avidin.

### 2.3.2 Cooperativity (and the Role of Enthalpy) in the "Chelate Effect"

Toone and co-workers have determined the enthalpy and entropy (using isothermal titration calorimetry) for the chelation of calcium(II) ion by the tetravalent carboxylate ligand, ethylenediaminetetraacetic acid (EDTA; Fig. 2.3b) [31]. Their results indicate that enthalpy drives the binding (in contrast to the classic explanation of entropy as the origin of the "chelate effect") and that the unbound state (unassociated metal ion and ligand) is coulombically destabilized relative to the fully bound complex of metal and ligand. This work emphasizes the necessity of considering the thermodynamics of both bound *and* dissociated states.

### 2.3.3 Oligovalency in the Design of Inhibitors to Toxins

Toxins in the $AB_5$ family are ideal protein targets for multivalent ligands: the toxins have five-fold symmetry and bind to monovalent sugars with low affinity ($K_d^{affinity}$~ mM; Fig. 2.3c) [36, 37]. Kitov, Bundle, and co-workers designed decavalent ligands, with a glucose scaffold, that bound to Shiga-like toxin with an enhancement of $10^7$ ($K_d^{avidity}$ ~ nM) [22, 32]. In related work, Hol, Fan, and co-workers targeted cholera toxin and heat-labile *E. coli* enterotoxin with both pentavalent and decavalent inhibitors with a pentacyclen scaffold; they also observed large enhancements in binding ($10^5$–$10^6$) [38–40].

### 2.3.4 Bivalency at Well Defined Surfaces (Self-assembled Monolayers, SAMs)

Reinhoudt, Huskens, and co-workers have studied the differences between bivalent binding in solution and at a structurally well defined surface [34, 41]. They examined the binding of a bivalent adamantane to a bivalent, soluble cyclodextrin, and to a self-assembled monolayer of cyclodextrin (Fig. 2.3d). The enhancement (β) in binding (relative to the monovalent interaction) was $10^3$ greater at a surface than in solution. They rationalized this difference in enhancement by postulating that the effective concentration (Section 2.4.3) of cyclodextrin in the vicinity of an unbound adamantane was much larger at the surface than in solution.

### 2.3.5 Polyvalency at Surfaces of Viruses, Bacteria, and SAMs

Polymeric ligands are effective at binding to the surfaces of viruses, bacteria, and SAMs [2, 4–10, 13–15, 42–44]. For example, we have examined the ability of poly-

mers presenting sialic acid to block the adhesion of influenza virus particles to erythrocytes (Fig. 2.3 e) [4–10]. We observed large enhancements on a per sialic acid basis (~$10^9$; $IC_{50}^{avidity}$ ~ pM relative to $IC_{50}^{affinity}$ ~ mM for monovalent sialic acid).

## 2.4 Theoretical Considerations in Multivalency

### 2.4.1 Survey of Thermodynamics

To understand the origins of the large enhancements of multivalent systems introduced in Sections 2.3.1–2.3.6, we need a theoretical model for multivalency. We introduce one here with the primary goal of demonstrating how the thermodynamics of multivalent systems differ from those of their monovalent components. A theoretical understanding will facilitate the design of tight-binding multivalent ligands to a variety of multivalent receptor targets.

The key thermodynamic principle in multivalency is that, ideally, the enthalpy of binding of a multivalent system is more favorable than that of the monovalent species, with little or no corresponding increase in the unfavorable translational and rotational entropy of binding (Fig. 2.2) [1, 45]. The enthalpy of interaction of a multivalent ligand with a multivalent receptor is, in principle, additive (the enthalpy of interaction of three ligands with a receptor is three times the enthalpy of interaction of a single ligand), while the entropy of interaction is not (since the three ligands are connected, association of one ligand with one receptor increases the local concentration of the other ligands and receptors, and decreases the unfavorable entropic penalty "paid" to bring ligands and receptors together).

In practice, several factors substantially complicate this simple picture: (i) strain in the oligovalent ligand and/or receptor, if the geometry of the ligands and receptors do not match, (ii) loss in entropy caused by constraining the linker in the receptor–ligand complex, and (iii) energetically favorable or unfavorable interactions between the linker and the receptor (e.g., the surface of the protein). We proceed to address these three factors.

### 2.4.2 Additivity and Multivalency

We take a simple theoretical approach (Fig. 2.4): we treat the individual receptor–ligand interactions between the two multivalent entities as additive and then include terms from multivalency that affect the free energy of binding favorably (e.g., "chelate effect" [45, 46], favorable contacts of the linker, positive cooperativity, statistical effects) or unfavorably (e.g., unfavorable contacts of the linker, loss in conformational entropy of the linker, negative cooperativity). The model accounts for complexes of the multivalent ligand and receptor with different numbers of receptor–ligand interactions; for example, a trivalent ligand can be bound

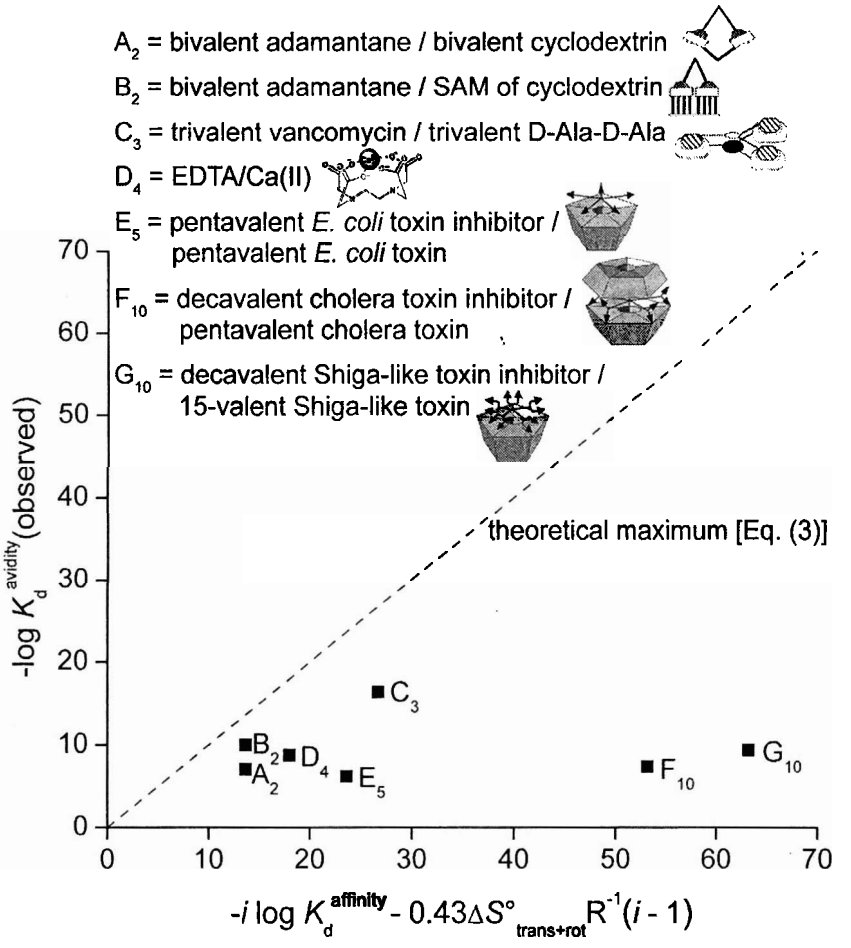
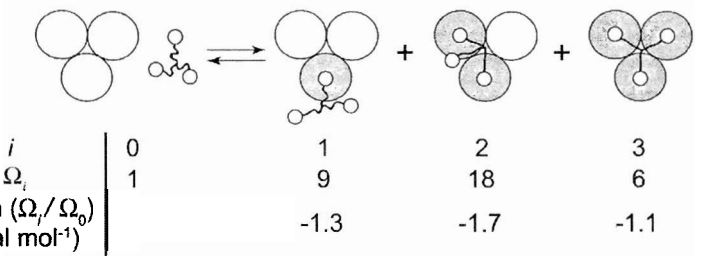



**Fig. 2.4**
Variation of $K_d^{avidity}$ with $K_d^{affinity}$ corrected for the valency of the interaction and for the translational and rotational entropic benefit of multivalency, for a number of experimental multivalent systems. The subscripts for the labeled points correspond to the number of interactions ($i$) between the two multivalent species. The abscissa results from the right-hand side of Eq. (3), assuming that the last four terms are negligible: interaction of the linker with the receptor, loss in conformational entropy of the linker, cooperativity between binding sites, and avidity entropy. The translational and rotational entropy was taken as –20 cal mol$^{-1}$ K$^{-1}$ ($-T\Delta S°_{trans+rot} \sim +6$ kcal mol$^{-1}$) for the loss in the modes of motion of the bound ligand relative to the receptor; this value is in the middle of those reported in the literature [17, 45]. The dashed line shows the theoretical maximum from multivalency [Eq. (3)]. All of the oligovalent ligands exhibit values of $K_d^{avidity}$ well below those expected from theory, perhaps due to large losses in the conformational entropy of the linker between ligands ($T\Delta S°_{conf}$). Further, values of the logarithm of $K_d^{avidity}$ cluster in a relatively narrow range and do not scale with the abscissa.

to a trivalent receptor by one ligand (two unbound ligands), by two ligands (one unbound ligand), or by three ligands (no unbound ligands; the fully-bound complex; Fig. 2.5). The model does not take into account aggregation of the multivalent receptor by the multivalent ligand (it assumes that the receptor is too dilute for this process to occur). We discuss aggregation of receptors in the context of multivalent toxins (Section 2.5.2.3) and antibodies (Section 2.7.3).



| $i$ | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| $\Omega_i$ | 1 | 9 | 18 | 6 |
| $-RT \ln(\Omega_i/\Omega_0)$ (kcal mol$^{-1}$) | | -1.3 | -1.7 | -1.1 |

**Fig. 2.5**
Graphical representation of $\Omega_i$ ("avidity entropy") as a function of the ligand–receptor interactions ($i$). The trivalent receptor is assumed to be a completely rigid assembly of three subunits; while the ligand has radial topology. The receptors are shaded gray when they are occupied by a ligand. For such a system, the number of degenerate states can be calculated from $\Omega_i = \frac{N!\,M!}{(N-i)!(M-i)!\,i!}$ where $N$ and $M$ are the valencies of the oligovalent ligand and receptor, respectively (here, $N = M = 3$); and $i$ is the number of ligand–receptor interactions [22]. The number of states reaches a maximum value at an occupancy ($i$) of less than the valency of the ligand ($N$). The free energy of binding for this term (difference in energy between the fully unassociated state and state with $i$ interactions) is virtually the same for all of the different occupancies.

Equation (3) gives the theoretical free energy of binding ($\Delta G^\circ{}_N(i)$; Fig. 2.4) for a multivalent ligand with $N$ ligands, as a function of the number ($i$) of ligands that are bound to receptors, where $i = 1 \ldots N$:

$$\Delta G^\circ_N(i) = i\Delta H^\circ_{\text{affinity}} - iT\Delta S^\circ_{\text{affinity}} + (i-1)T\Delta S^\circ_{\text{trans+rot}} + (i-1)\Delta H^\circ_{\text{linker}} - (i-1)T\Delta S^\circ_{\text{conf}} + (i-1)\Delta G^\circ_{\text{coop}} - RT\ln(\Omega_i/\Omega_0) \quad (3)$$

The first term ($i\Delta H^\circ_{\text{affinity}}$) is the product of the number of bound ligands ($i$) for the particular receptor–ligand state and the monovalent enthalpy of binding ($\Delta H^\circ_{\text{affinity}}$). The second term ($-iT\Delta S^\circ_{\text{affinity}}$) is analogous to the first but deals with the monovalent entropy of binding ($-T\Delta S^\circ_{\text{affinity}}$). The sum of these first two terms ($i\Delta H^\circ_{\text{affinity}} - iT\Delta S^\circ_{\text{affinity}}$) is the free energy of binding that would be observed if the $i$ receptor–ligand interactions occurred independently (i.e., if there were no effect of oligovalency). The third term [$(i-1)T\Delta S^\circ_{\text{trans+rot}}$] deals with the classic "chelate effect" [45, 46] and conceptually is the center of multivalency: the unfavorable translational and rotational entropy of binding is approximately the same for the multivalent interaction as for the monovalent one. We note that the translational and rotational entropies show weak (logarithmic) dependences on the mass and dimensions of particles [1, 47]. This weak dependence justifies the assumption that the monovalent and multivalent interactions have equal translational and rotational entropies. The correlation between enthalpy of binding and translational and rotational entropy of binding (enthalpy/entropy compensation) [48–50] will, however, complicate this analysis. We discuss this effect in Section 2.4.6. The effect of the term $(i-1)T\Delta S^\circ_{\text{trans+rot}}$ is to "add back" the unfavorable entropy of complexation for all receptor–ligand interactions but one [i.e., for $(i-1)$ interactions]. The fourth term [$(i-1)\Delta H^\circ_{\text{linker}}$] deals with any enthalpic contacts (favorable or un-

favorable) between the linker(s) and the oligovalent receptor. This simple analysis, summarized by Eq. (3), assumes that each receptor–linker interaction occurs with the same enthalpy ($\Delta H^{\circ}_{\text{linker}}$) [51, 52]. The fifth term ($-(i-1)T\Delta S^{\circ}_{\text{conf}}$) accounts for the loss in conformational entropy of the linker(s) between the ligands (and between protein subunits, if applicable; Section 2.4.5) [17, 46, 53]. The sixth term [$(i-1)\Delta G^{\circ}_{\text{coop}}$] addresses effects of cooperativity between binding sites (either between protein subunits or individual ligands). Such effects originate from the influence of one binding event on subsequent ones (Section 2.4.4) [54]. This term is usually near zero (i.e., the individual binding sites behave independently). The final term is a statistical factor dealing with the degeneracy ($\Omega_i$) for each receptor–ligand complex (each with a different number of receptor–ligand interactions, $i$; Fig. 2.5). Kitov and Bundle discussed the importance of this term (which they define as the "avidity entropy") in multivalent systems [22].

Our assumption for *bivalent* and *trivalent* systems, in which the number of ligands is equal to the number of receptors ($N = M$), is that all ligands (of the oligovalent ligand) are bound to receptors (on the same oligovalent receptor) in the only bound state of the complex [i.e., $\Delta G^{\circ}_{N}(N) \ll \Delta G^{\circ}_{N}(N-1)$, $\Delta G^{\circ}_{N}(N-2),\ldots,$ $\Delta G^{\circ}_{N}(1)$]. This assumption is not valid for higher *oligovalent* or *polyvalent* systems. Kitov and Bundle proposed a more general but less convenient definition than ours (see Section 2.2.1) [22]. Under the assumption above, Eq.(3) simplifies to Eq. (4) for the only populated state of a bivalent system ($i = 2$):

$$\Delta G^{\circ}_{2}(2) = 2\,\Delta H^{\circ}_{\text{affinity}} - 2\,T\Delta S^{\circ}_{\text{affinity}} + T\Delta S^{\circ}_{\text{trans+rot}} + \Delta H^{\circ}_{\text{linker}} - T\Delta S^{\circ}_{\text{conf}} + \Delta G^{\circ}_{\text{coop}} - RT\ln 2 = \Delta G^{\circ}_{\text{avidity}} \quad (4)$$

The free energy of binding for this only occupied state for the receptor-ligand complex is equal to the avidity free energy ($\Delta G^{\circ}_{\text{avidity}}$; Fig. 2.2).

Figure 2.4 shows a comparison of observed and theoretical $K_{\text{d}}^{\text{avidity}}$ as a function of the number of receptor–ligand interactions ($i$) and $K_{\text{d}}^{\text{affinity}}$ for a number of multivalent systems. For clarity in the plot, only the first three terms in Eq. (3) are included in the abscissa (Fig. 2.4). All of the experimental systems show avidities much lower than that expected from the theory (Section 2.8).

### 2.4.3
### Avidity and Effective Concentration ($C_{\text{eff}}$)

Several investigators [34, 41, 55, 56] have discussed avidity in terms of the effective concentration ($C_{\text{eff}}$) of an unbound ligand near an unbound receptor when the oligovalent receptor and ligand are bound at another site (Fig. 2.6). Lees and coworkers [56] estimated $C_{\text{eff}}$ for the binding of an oligovalent ligand to a rigid oligovalent receptor from polymer theory [57, 58]. They assumed that the linker between the ligands was subject to random Gaussian chain statistics and that $C_{\text{eff}}$ was proportional to the probability that the distance between the unbound ligand and the bound ligand (the "ends" of the polymer) was equal to the distance between receptors (Fig. 2.6c). The application of random-walk statistics requires

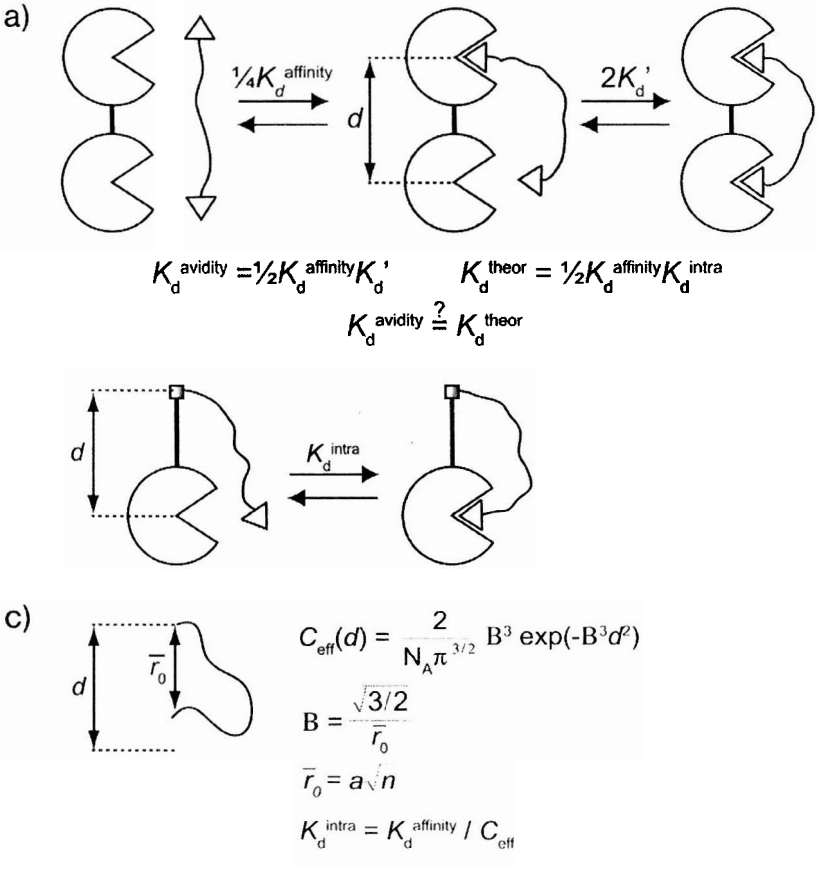



**Fig. 2.6**
Graphical representation illustrating a method to assess cooperativity in multivalent systems. (a) The binding of an oligovalent ligand to an oligovalent receptor (with two subunits which are a distance $d$ apart) can be conceptualized as occurring in two steps: an intermolecular one and an intramolecular one. $K_d^{avidity}$ is obtained experimentally, while $K_d^{theor}$ is obtained using Eq. (5) and requires independent measurements of $K_d^{affinity}$ and $K_d^{intra}$. A comparison of $K_d^{avidity}$ and $K_d^{theor}$ allows the assessment of cooperativity between the two receptors. (b) A hypothetical reaction to measure the dissociation constant for intramolecular binding ($K_d^{intra}$). The long, "unphysical" pole is shown to maintain the fixed distance ($d$) used for the separation between protein subunits in (a). (c) A method to estimate $K_d^{intra}$ using effective concentration ($C_{eff}$): the probability that the ends of a polymer (at variable distance of $r_0$) will be the appropriate distance ($d$) apart. The equations assume a random-coil model and show the dependence of the effective concentration on the distance between binding sites ($d$) and the number of subunits ($n$) in the linker. $N_A$ is Avogadro's number. The value $a$ is a constant that characterizes the stiffness of different chains and varies between 1.5 and 5.5 for most linkers [57]. Flexible linkers, such as oligo(ethylene glycol) are characterized by higher values of $a$ [59].

that the linker behaves as a random coil polymer [57]. If the linker were too short, this condition would not be met, and the two "ends" would not rotate freely relative to one another [57]. Steric effects along the backbone between non-adjacent units of the linker (e.g., transannular strain in the linker in the fully bound receptor–ligand complex) would also invalidate the assumption of Gaussian chain statistics [58]. In addition, contacts between the linker and the oligovalent receptor

could effectively shorten the linker and result in a higher value for $C_{eff}$ than that predicted by the theory.

### 2.4.4 Cooperativity is Distinct from Multivalency

Cooperativity occurs when the binding of one ligand to a receptor affects the binding (that is, the dissociation constant) of additional ligands to the same receptor [54]. Cooperativity has been defined rigorously for the binding of multiple, *monovalent* ligands to a multivalent receptor (usually a protein). In biochemistry, a cooperative interaction occurs when the binding of one monovalent ligand to one site of a multivalent protein results in a change in the conformation of the protein (or stabilizes that alternative conformation) that extends to other binding sites; this conformational change affects the binding of subsequent ligands to the protein [60, 61]. In positive cooperativity, $K_{d,n}$, the dissociation constant for the binding of a ligand to a receptor already bound to $n$ ligands, is smaller than $K_{d,n-1}$, the dissociation constant for the binding of a ligand to the receptor bound to $n-1$ ligands (after correction of both values for statistical factors). In negative cooperativity, $K_{d,n}$ is greater than $K_{d,n-1}$. In a system with no cooperativity (i.e., independent binding sites), $K_{d,n}$ is equal to $K_{d,n-1}$.

Ercolani discussed a method of assessing cooperativity in multivalent systems (Fig. 2.6a) by comparing $K_d^{avidity}$ with $K_d^{theor}$ [54]. $K_d^{theor}$ is defined in Eq. (5):

$$K_d^{theor} = cK_d^{affinity}(K_d^{intra})^{n-1} \tag{5}$$

Here, $c$ is a statistical factor and $K_d^{intra}$ describes a hypothetical intramolecular binding process (Fig. 2.6b) [54]. If $K_d^{avidity}$ is less than $K_d^{theor}$, positive cooperativity is at work. If $K_d^{avidity}$ is greater than $K_d^{theor}$, negative cooperativity is occurring in the system. If the two values ($K_d^{avidity}$ and $K_d^{theor}$) are equal, the system is non-cooperative. Several investigators proposed using a theoretical estimate of $C_{eff}$ to estimate $K_d^{intra}$ (Fig. 2.6c) [34, 41, 55, 56]; an assessment of cooperativity could then follow the method of Ercolani [Eq. (5)].

The design of a suitable intramolecular reference reaction to measure $K_d^{intra}$ directly can be difficult in biological systems. Estimating $C_{eff}$ for an oligovalent ligand faces the difficulties described in Section 2.4.3. Given these complications, we propose that a comparison of the experimentally observed enthalpy terms from Eq. (3) [$\Delta H^{\circ}_{theor}$, defined in Eq. (6)] with the observed enthalpy of the multivalent interaction ($\Delta H^{\circ}_{avidity}$) can be used to assess cooperativity in multivalent systems:

$$\Delta H^{\circ}_{theor} = i\Delta H^{\circ}_{affinity} + (i-1)\Delta H^{\circ}_{linker} \tag{6}$$

where the terms are as defined for Eq. (3). If $\Delta H^{\circ}_{avidity}$ is more favorable than $\Delta H^{\circ}_{theor}$, the system is positively cooperative. If $\Delta H^{\circ}_{avidity}$ is less favorable than $\Delta H^{\circ}_{theor}$, the system is negatively cooperative (perhaps due to strain from a sub-op-

timal linker; Section 2.6.3). If $\Delta H^{\circ}_{\text{avidity}}$ is equal to $\Delta H^{\circ}_{\text{theor}}$ the system is non-cooperative (and the two binding events occur independently). This approach avoids the difficulties with the approach based on $K_d^{\text{intra}}$ [Eq. (5); which uses only free energies of binding], because it removes the entropy of binding [the primary contributor to multivalency; Eq. (3)] from the examination of the multivalent system. The application of Eq. (6) requires accurate measurements of the enthalpy and entropy of binding for complexation; and the best technique for such measurements is isothermal titration calorimetry (Section 2.5.1). To date, there have been no reported *multivalent biological* examples (where both the ligand and receptor are multivalent) in which cooperativity contributes to avidity.

### 2.4.5
### Conformational Entropy of the Linker between Ligands

We [1, 53] and others [17, 32, 38, 40, 46] suggested that the linker for the oligovalent ligand should be rigid in order to minimize the loss in conformational entropy [$T\Delta S^{\circ}_{\text{conf}}$ in Eqs. (3), (4)] that occurs upon binding the receptor (Fig. 2.7). The intuitive argument is that flexible linkers have more entropy to lose (more accessible conformational states) upon association than rigid ones, and thus should be avoided to achieve tight binding. A rough estimate of the conformational entropy that is lost upon complexation for a flexible linker is $RT \ln 3 \sim 0.7$ kcal mol$^{-1}$ per three-fold rotor (freely rotating bond) of the linker (Fig. 2.7). The situation is, in fact, substantially more complex than this simple analysis might suggest: for instance, residual mobility of the linker in the bound complex, and a pre-organized ligand (and linker) when unassociated with receptor, would both reduce the loss in conformational entropy. We discuss design principles of ligands more extensively in Section 2.6.3, together with alternative models for linker flexibility. One of these models gives a much lower loss in conformational entropy of a flexible linker than that predicted here.

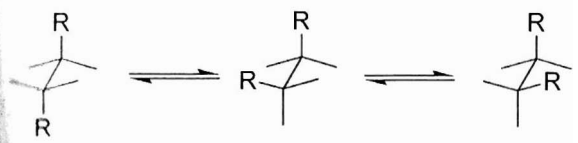



**Fig. 2.7**
Conformational entropy about rotors. We assume that the three conformational states above are degenerate. When one conformation is populated exclusively on binding of a protein to a ligand with this linker, the loss in conformational entropy (at $T = 298$ K) will be –0.7 kcal mol$^{-1}$ per rotor (freely rotating bond) of the linker ($-T\Delta S^{\circ}_{\text{conf}} \sim m RT \ln 3$, where $m$ is the number of rotors in the linker).

### 2.4.6
### Enthalpy/Entropy Compensation Reduces the Benefit of Multivalency

Our model assumes that the primary benefit of multivalency is the entropic enhancement of linking the ligands together [third term in Eq. (3)], so that the rotational and translational entropy of binding for a multivalent ligand is the same as for the component monovalent ligand ($T\Delta S^{\circ}_{trans+rot}$). The complication of using Eq. (3) quantitatively to estimate free energies of binding of multivalent systems is that more exothermic interactions are correlated with greater rotational and translational entropic costs of association, a phenomenon known as enthalpy/entropy compensation (EEC) [48–50]. This greater entropic cost is attributed to residual mobility of the ligand in the receptor–ligand complex [48, 49, 62]. A multivalent interaction (with no cooperativity between binding sites) has an enthalpy of binding that is the sum of the monovalent interactions [Eq. (6)]. EEC predicts that this multivalent interaction will have a greater entropic cost than the monovalent interaction ($-T\Delta S^{\circ}_{trans+rot}{}^{avidity} > -T\Delta S^{\circ}_{trans+rot}$) and not the same entropic cost as the monovalent interaction [predicted by Eq. (3)]. This compensation will decrease the magnitude of the free energy of binding (increase $K_d^{avidity}$) relative to that expected from Eq. (3). This greater entropic cost of association does not completely compensate for the more favorable enthalpy of binding in most cases; the free energy of binding does, generally, become more favorable with more favorable enthalpy [49, 62]. Dunitz presented a simple theoretical model for EEC [48]. Williams and co-workers discussed EEC in the context of residual mobility of the *receptor* as well as the ligand in the receptor–ligand complex [49].

## 2.5
## Representative Experimental Studies

### 2.5.1
### Experimental Techniques Used to Examine Multivalent Systems

A number of experimental techniques have been used to study multivalent systems in solution (ITC) and at surfaces (SPR, ELISA, hemagglutination). We summarize these techniques briefly in this section.

#### 2.5.1.1 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) measures heats of association for receptor–ligand complexation, as one component is titrated into the other. Analysis of these heats as a function of the concentration of the ligand relative to the receptor yields values for the enthalpy of binding ($\Delta H^{\circ}$) and the dissociation constant ($K_d$; when this value is the range of μM–nM) [63, 64]. Turnbull and Daranas suggested that ITC can also determine $K_d$, but perhaps not $\Delta H^{\circ}$, for lower affinity (~ mM) systems [65]. ITC can thus determine both the free energy and the enthalpy of bind-

ing; and the entropy of binding can be determined from these two values ($T\Delta S^\circ = \Delta H^\circ - \Delta G^\circ$). Because ITC is carried out at one temperature (and so without concerns for changes in protein structure with temperature, and without assuming the change in heat capacity upon complexation is independent of temperature), it generates much more reliable thermodynamic information for biological systems than does van't Hoff analysis, which requires measurements at different temperatures [66–69]. For thermodynamic analysis of multivalent systems, the accuracy provided by ITC is essential in understanding both the entropic and enthalpic contributions to binding, in determining the thermodynamic advantage gained from multivalency (relative to monovalency), and in assessing the cooperativity of the system (Section 2.4.4).

#### 2.5.1.2 Surface Plasmon Resonance Spectroscopy

Surface plasmon resonance (SPR) [70–72] spectroscopy provides both kinetic and thermodynamic ($K_d$) data for the binding of small molecules and proteins to surfaces. One component of the binding interaction is covalently attached to the surface; and the refractive index near the surface (proportional to the amount of material adsorbed to the surface) is monitored as the other component is flowed across the surface. Surfaces that have been used include self-assembled monolayers (SAMs) [34, 41, 44, 72, 73], which permit rigorous physical–organic characterization of protein–ligand interactions, because the structure of the monolayer at the surface is well defined [73], and the matrices of gels (e.g., dextran) [74–76] which allow for high levels of binding (and thus a stronger signal than with SAMs) but with ambiguities concerning the partitioning of the soluble component into the gel.

#### 2.5.1.3 Surface Assays Using Purified Components (Cell-free Assays)

There are several other techniques (e.g., ELISA) for measuring binding of multivalent ligands or receptors to surfaces using *in vitro* assays [8, 32, 77]. These assays provide parameters (e.g., $IC_{50}$) for characterizing binding that are more empirical than $K_d^{avidity}$, because they may measure more complex processes. For instance, measurement of the binding of multivalent ligands to multivalent toxins is often based on the initial non-specific adsorption of the toxin to a microwell plate [12, 78]. The surface-bound toxin is then allowed to equilibrate with the multivalent ligand and a monovalent competitor linked to a reporter molecule; the reported signal is proportional to the concentration of free toxin (i.e., toxin not bound to the multivalent ligand).

#### 2.5.1.4 Cell-based Surface Assays

Some surface assays (e.g., hemagglutination [79], optical tweezers [10, 80], fluorescent cells attached to surfaces [81]) are based on whole cells and thus have the benefit that they are measuring biological activity at authentic biological surfaces.

Hemagglutination assays involve observing the agglutination of erythrocytes by bacteria, viruses, or microspheres into a gel, and determining the concentration of multivalent ligand required to inhibit this process (i.e., to allow the erythrocytes to sediment into a compact pellet). Additional examples of cell-based assays are shear-flow experiments. The assay of Kiessling and co-workers involves incubating selectin-transfected cells (models for leukocytes) with different concentrations of a multivalent ligand and then microscopically monitoring the rolling of the cells on substrates coated with ligands to selectin [2, 3]. This assay system is more physiologically relevant for the inhibition of attachment of leukocytes to endothelial cells than static cell-free assays, which do not involve shear.

### 2.5.2 Examination of Experimental Studies in the Context of Theory

We now examine the examples presented earlier (Section 2.3) in the context of theoretical aspects of multivalency (introduced in Section 2.4).

#### 2.5.2.1 Trivalency in Structurally Simple Systems

The thermodynamic stability of the trivalent vancomycin·D-Ala-D-Ala system (Fig. 2.3a) seems to derive, in large part, from the geometric match between the two components [29, 30]. The relatively rigid scaffolds (aromatic rings) and linkers [*p*-substituted aromatic rings for the vancomycin derivative and short alkyl chains (butyl) for D-Ala-D-Ala] make this match possible. ITC measurements revealed that the enthalpy of binding ($\Delta H^{\circ}_{\text{avidity}} = -40$ kcal mol$^{-1}$) was approximately three times that of the monovalent interaction [$\Delta H^{\circ}_{\text{affinity}} = -12$ kcal mol$^{-1}$, as predicted by theory; Eqs. (3), (6)] [29]. The unfavorable entropy of binding ($-T\Delta S^{\circ}_{\text{avidity}} =$ 18 kcal mol$^{-1}$), however, was approximately 4.5 times that of the monovalent interaction ($-T\Delta S^{\circ}_{\text{affinity}} = 4.1$ kcal mol$^{-1}$); and the theoretical maximum for avidity would have the entropy of binding the same for both processes [Eq. (3)]. Using the semi-quantitative analysis of the binding of monovalent D-Ala-D-Ala to vancomycin by Williams et al. [82], we estimated a conformational entropy loss ($-T\Delta S^{\circ}_{\text{conf}}$) of ~ 41 kcal mol$^{-1}$ upon complexation [30]. Averaging this entropy over the 27 total rotors of the trivalent molecules that were frozen upon complexation, gave $-T\Delta S^{\circ}_{\text{conf}} \sim 1.5$ kcal mol$^{-1}$ per rotor frozen upon complexation (in good agreement with the estimate of Page and Jencks [46], but higher than a simple theoretical estimate of 0.7 kcal mol$^{-1}$; Section 2.4.5). The remarkable result was that the loss in conformational entropy was almost exactly offset by the gain from translational and rotational entropy of linking the ligands (and receptors) together ($-T\Delta S^{\circ}_{\text{conf}} \sim -2T\Delta S^{\circ}_{\text{trans,rot}}$) in the most likely model. Even though the observed enhancement in binding for this system fell far short (by a factor of ~ $10^{11}$) of the maximum expected theoretically [Eq. (3), Fig. 2.4], it remains one of the tightest-binding examples of a low molecular weight ligand–receptor interaction in water. This system also demonstrates that large enhancements ($\beta = 10^{11}$) are possible, even if the theoretical, maximum benefit of multivalency is not obtained.

#### 2.5.2.2 Cooperativity (and the Role of Enthalpy) in the "Chelate Effect"

Toone and co-workers have interpreted their thermodynamic studies of the chelation of Ca(II) ion by the tetravalent chelating agent EDTA to indicate that the high avidity binding is primarily due to a favorable enthalpy, not a favorable entropy [31]. This result is in contrast to the theoretical treatment [Eq. (3)] that predicts an entropic origin for the increased avidity of multivalent systems. The monovalent interaction (acetate binding to Ca(II)) has an unfavorable enthalpy ($\Delta H^{\circ}_{affinity}$ = +1.3 kcal mol$^{-1}$; $-T\Delta S^{\circ}_{affinity}$ = −3.0 kcal mol$^{-1}$) compared to the favorable value for tetravalent EDTA ($\Delta H^{\circ}_{avidity}$ = −5.3 kcal mol$^{-1}$; $-T\Delta S^{\circ}_{avidity}$ = −6.7 kcal mol$^{-1}$). According to our definition of cooperativity (Section 2.4.4), this interaction is therefore an example of cooperativity of the ligand sites [$\Delta H^{\circ}_{avidity} < 4\Delta H^{\circ}_{affinity}$; Eq. (6)]. The investigators hypothesized that the origin of this cooperative behavior is a relief of charge–charge repulsion [83–85] in the unbound state of EDTA upon binding to Ca(II). This charge–charge repulsion may also make the unbound state of the ligand rigid; and this restricted motion would lead to an insignificant loss of conformational entropy (Section 2.4.5) upon binding to Ca(II) [$-T\Delta S^{\circ}_{conf} \sim 0$ kcal mol$^{-1}$; Eq. (3)], since the ligand is also rigid when fully associated with the calcium ion. Indeed, the investigators observed similar entropies of binding for a series of homologous ligands with equivalent valency but with different numbers of rotors between the ligands [31].

#### 2.5.2.3 Oligovalency in the Design of Inhibitors of Toxins

Kitov, Bundle, Hol, Fan, and co-workers designed penta- and decavalent glycoside ligands for $AB_5$ family toxins, including Shiga-like toxin, cholera toxin, and heat labile *E. coli* enterotoxin [22, 32, 38–40]. The ligands were designed with approximate five-fold symmetry, to match that of the toxins. The linkers in these designs are long (~12 ethylene glycol units) and flexible; these characteristics ensure that the pendant glycosides can bind to the receptors around the periphery of the toxin (Fig. 2.3c). X-ray crystal structures of both Shiga-like [32] and cholera [39, 40] toxins bound to the penta- and decavalent ligands were unable to resolve the central core (scaffold) and linkers of the ligands; these results suggest a highly disordered, flexible region. While theory predicts that long, flexible linkers would be subject to large losses of conformational entropy ($-T\Delta S^{\circ}_{conf}$) upon binding (Section 2.4.5), the toxin ligands still bind with high avidities (values of β up to $10^7$). We discuss this apparent contradiction in Section 2.6.3. The observed avidities are much lower than the maxima expected from theory, however (Fig. 2.4); and so large losses in conformational entropy could be making unfavorable contributions to the avidities. There are no calorimetric data for the binding of these penta- and decavalent ligands to their toxin receptors, so we are not able to comment on the relative enthalpic and entropic (e.g., $-T\Delta S^{\circ}_{conf}$) contributions to the avidity of binding.

The work with decavalent ligands introduces another, important design principle: using one multivalent receptor (protein) to pre-organize and present a multivalent ligand to another multivalent receptor (Fig. 2.3c). Crystal structures and

dynamic light-scattering experiments demonstrate that the decavalent inhibitors of both Shiga-like [32] and cholera [39] toxin dimerize the pentameric toxins. Fan, Hol, and co-workers extended this strategy to form heterodimers of two different pentavalent proteins (cholera toxin and human serum amyloid P component) using a hetero-bivalent ligand [86]. The hetero-bivalent molecule and one protein were pre-incubated, forming a new pentavalent ligand that was then introduced to the other protein. They observed enhancements (β) of up to three orders of magnitude in the value of $IC_{50}$ (Section 2.5.1) for the binding of the complex of hetero-bivalent ligand and cholera toxin to surface-bound human serum amyloid.

In ELISA-like assays, where only 1:1 binding is possible, the decavalent inhibitors bound to the toxins with a higher avidity (by a factor of 10–100) than do the corresponding pentavalent ligands [22, 39]. A possible explanation for this observation is that the avidity entropy [Eq. (3), Fig. 2.5] becomes important when the decavalent ligands bind to a pentavalent receptor [22].

#### 2.5.2.4 Bivalency in Solution and at Well Defined Surfaces (SAMs)

Reinhoudt, Huskens, and co-workers studied the binding of a bivalent adamantane derivative (bivalent ligand), both to a bivalent cyclodextrin derivative (by ITC), and to a SAM displaying a saturating coverage of cyclodextrins (by SPR; Fig. 2.3 d) [34].

In solution, calorimetric data demonstrated that the enthalpy of binding of the bivalent adamantane to bivalent cyclodextrin (CD) was approximately two-fold that for bivalent adamantane to monovalent CD ($\Delta H^{\circ}_{avidity} = -14.8$ kcal mol$^{-1}$, $\Delta H^{\circ}_{affinity} = -7.0$ kcal mol$^{-1}$) [34]; a result that indicated that the binding sites were non-cooperative [Eq. (6)]. The entropy of binding to the bivalent CD was significantly more unfavorable than that for the monovalent CD ($-T\Delta S^{\circ}_{avidity} = +5.1$ kcal mol$^{-1}$, $-T\Delta S^{\circ}_{affinity} = +0.6$ kcal mol$^{-1}$), and much more unfavorable than expected from theory [Eq. (3) predicts the same entropic cost for the bivalent and monovalent associations for the theoretical maximum of avidity]; and this unfavorable term reduced the enhancement (β) to ~300 from the maximum enhancement from theory of ~$10^9$ (Fig. 2.4). Both the bivalent adamantane and bivalent CD contained linkers of oligo(ethylene glycol), which is expected to be very flexible and result in a large loss in conformational entropy ($-T\Delta S^{\circ}_{conf} > 0$) upon association (Section 2.4.5, Fig. 2.7).

The investigators observed that the bivalent adamantane bound more tightly (by ~$10^3$) to a SAM displaying a saturating coverage of CD than to the bivalent CD in solution [34]. They calculated the effective concentration ($C_{eff}$; Section 2.4.3) for binding in each (in solution, $C_{eff} \geq 1.8$ mM; at the surface, $C_{eff} \geq 200$ mM); this $C_{eff}$ ~$10^2$ greater at the surface than in solution is consistent with the observation that binding at a surface is tighter than in solution. Figure 2.3 d shows the difference in $C_{eff}$ graphically: there are more cyclodextrin receptors available on the surface of the SAM than in solution, within the probing volume of the uncomplexed adamantane (defined by the average end-to-end distance between the complexed and uncomplexed adamantanes). The investigators

extended this work to the binding of ligands with different valencies (dendrimers with pendant adamantane or ferrocene moieties, and polymers displaying adamantane) to cyclodextrin SAM surfaces [41, 87–89] They did not, however, examine the binding of oligovalent adamantanes to surfaces with different mole fractions of cyclodextrin.

### 2.5.2.5 Polyvalency at Surfaces (Viruses, Bacteria, and SAMs)

Polymers are useful as polyvalent scaffolds in three circumstances: (i) when it is technically difficult, impractical, or undesirable to design a multivalent ligand that matches a multivalent receptor geometrically, (ii) when the objective of the association is not just to fill the active site of the receptors with ligand, but to serve some other function (e.g., putting a layer of polymer on a surface to prevent adhesion, presenting a second type of ligand at a surface, attaching a hapten or a fluorophore to the surface, or delivering a drug compound to the cell) [4–10, 14, 15, 43, 90, 91] (Fig. 2.8), and (iii) when one wishes to present multiple types of ligands, in a polyvalent fashion, to one multivalent species (e.g., a cell surface with multiple types of receptors [5, 7]) [1, 16].

In one example, we designed and synthesized polyacrylamides with side-chains of sialic acid (Fig. 2.3e); these polymers inhibited the binding of influenza virus to erythrocytes (an interaction that occurs via the binding of hemagglutinin, a capsid coat protein on influenza virus, to sialic acid groups at the termini of the oligosaccharides on the surface of erythrocytes) [4–10, 90]. In cell-based assays (hemagglutination), the best of these polymers displayed enhancements (β) of $10^9$ (based

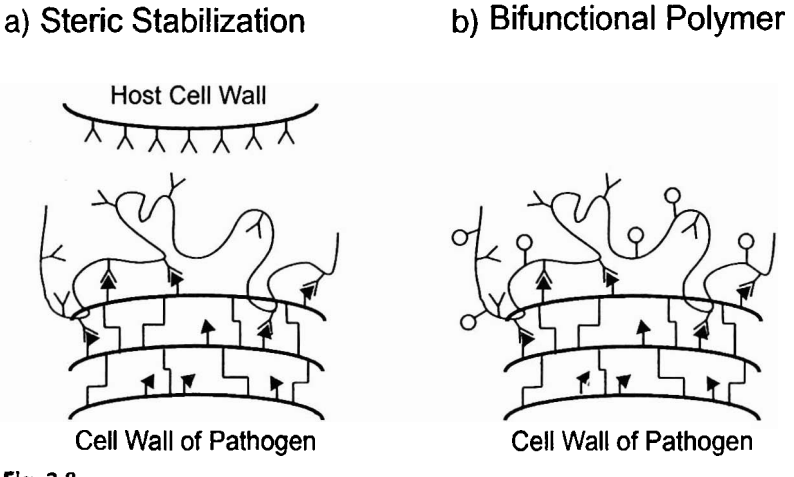



**Fig. 2.8**
Schematic representations of two different applications of polymers in binding to the cell walls of pathogens. (a) The polymer binds to the pathogen and prevents its association with a host cell through steric repulsion (steric stabilization). (b) A polymer presenting two different types of functional elements (bifunctional polymer) binds to the surface of a pathogen (mediated by one functionality, "Y") and presents the other functionality (o) at the surface of the pathogenic cell.

$$\Delta G^{\circ}_{\text{avidity}}(i) \approx i\Delta G^{\circ}_{\text{affinity}} + (i-1)T\Delta S^{\circ}_{\text{trans+rot}}$$
$$\approx i\Delta G^{\circ}_{\text{affinity}} + c(i-1)\Delta H^{\circ}_{\text{affinity}} \quad (7)$$

For clarity, Eq. (7) omits contributions of conformational entropy ($-T\Delta S^{\circ}_{\text{conf}}$), cooperativity between binding sites ($\Delta G^{\circ}_{\text{coop}}$), and the avidity entropy [$-RT \ln(\Omega_i/\Omega_0)$] to the avidity. From the concept of enthalpy/entropy compensation (Section 2.4.6), binding events with more favorable enthalpies of binding are associated with more unfavorable translational and rotational entropies of association; this idea is shown by relating $T\Delta S^{\circ}_{\text{trans+rot}}$ to $\Delta H^{\circ}_{\text{affinity}}$ by the constant $c$ in Eq. (7). The more unfavorable entropy does not completely compensate for the more favorable enthalpy, and so $0 < c < 1$. Because this entropic term is "added back" in Eq. (7), multivalent interactions that are based on monovalent interactions with more favorable enthalpies of binding gain more in terms of free energy (more favorable) than those based on monovalent interactions with less favorable enthalpies.

## 2.6.2 Choice of Scaffold for Multivalent Ligands

The spacing of receptors in an oligovalent protein is defined by the system (as it occurs naturally). For this reason, our design rules focus on the multivalent ligand; specifically, we discuss the scaffold for tethering the ligands together (Section 2.6.2), and the linker to connect the ligands to the scaffold (Section 2.6.3; Fig. 2.9a). We do not attempt to discuss design of the ligands themselves and simply assume that the "best" choice of ligand is based on some combination of availability, ease of modification, affinity, and other biological and physical (e.g., solubility, stability) properties.

### 2.6.2.1 Scaffolds for Oligovalent Ligands

The design of oligovalent ligands often requires a scaffold that serves to present the ligands (attached by linkers) to the oligovalent receptor (Fig. 2.9a). Figure 2.9b shows some of the common scaffolds that have been used to connect ligands in the design of oligovalent ligands [16]. A common approach is to use a rigid central element to minimize the loss in conformational entropy upon complexation (Section 2.4.5). Most of the ligand scaffolds are planar, to facilitate the matching of distances and angles in the target oligovalent receptor. The scaffold should also match the symmetry of the target receptor (e.g., the design of a pentavalent ligand starting with a pentacyclen scaffold and five pendant ligands in studies of binding to pentavalent cholera toxin [36, 38, 40]). A mismatch in these design elements with the biological target will result in strain in the receptor–ligand interactions and could result in steric repulsion between scaffold and receptor; both of these effects will reduce the avidity.

In certain cases (primarily bivalent ligands), the ligands have been directly connected to one another via a linker without a scaffold (Fig. 2.9a) [100, 102, 104,

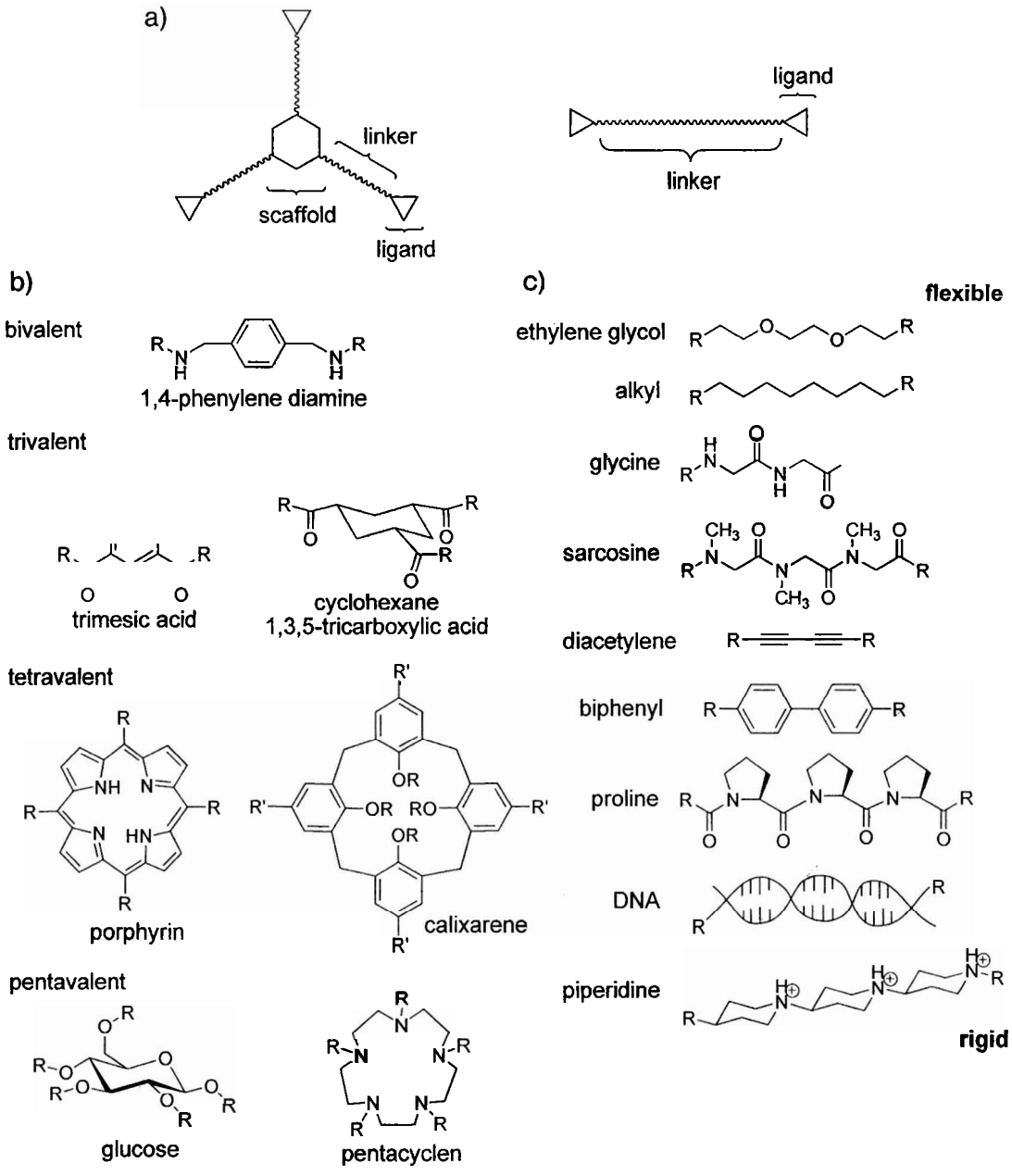
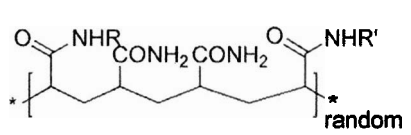



**Fig. 2.9**
Scaffolds and linkers for oligovalent ligands [16]. (a) Schematic showing the different parts of an oligovalent ligand: the scaffold, linkers, and ligands. The right-most structure shows a bivalent ligand that does not contain a scaffold. (b) Representative examples of scaffolds that have been used in oligovalent ligands of different valency: bivalent (1,4-phenylene diamine [93]), trivalent (trimesic acid [29, 30] and 1,3,5-cyclohexane tricarboxylic acid [94]), tetravalent (porphyrin [95] and calixarene [27]), and pentavalent (glucose [22, 32] and pentacyclen [38]). The only reported examples of oligovalent ligands containing scaffolds of 1,3,5-cyclohexane tricarboxylic acid or calixarene are studies in organic solvents. (c) Representative examples of linkers that have been used in oligovalent ligands: ethylene glycol [22, 32, 38], alkyl [29, 30], glycine [96], sarcosine [97], diacetylene [98], biphenyl [99], proline [100, 101], DNA [102], and piperidine [103]. The bivalent ligand containing the diacetylene linker was studied in an organic solvent (no aqueous examples are reported). A more expansive collection can be found in the book by Choi [16].

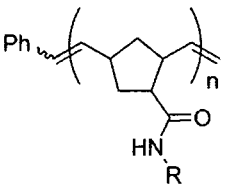
105]. This design maximizes the avidity entropy (Section 2.4.2) because the number of states for the fully associated receptor–ligand complex is at a maximum (there are more ways to arrange the receptor–ligand complex than when the ligands are bound to a rigid scaffold). Kitov and Bundle discussed the influence of topology of the oligovalent ligand on avidity entropy [22]. There is a trade-off between minimizing the loss in conformational entropy (Section 2.4.5) and maximizing the avidity entropy in selection of a scaffold.

### 2.6.2.2 Scaffolds for Polyvalent Ligands

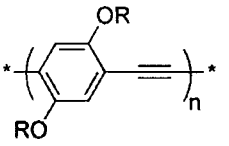
The two common types of scaffolds for polyvalent ligands are linear, random-coil (e.g., polymers) or approximately spherical (e.g., dendrimers; Fig. 2.10) [16]. Un-



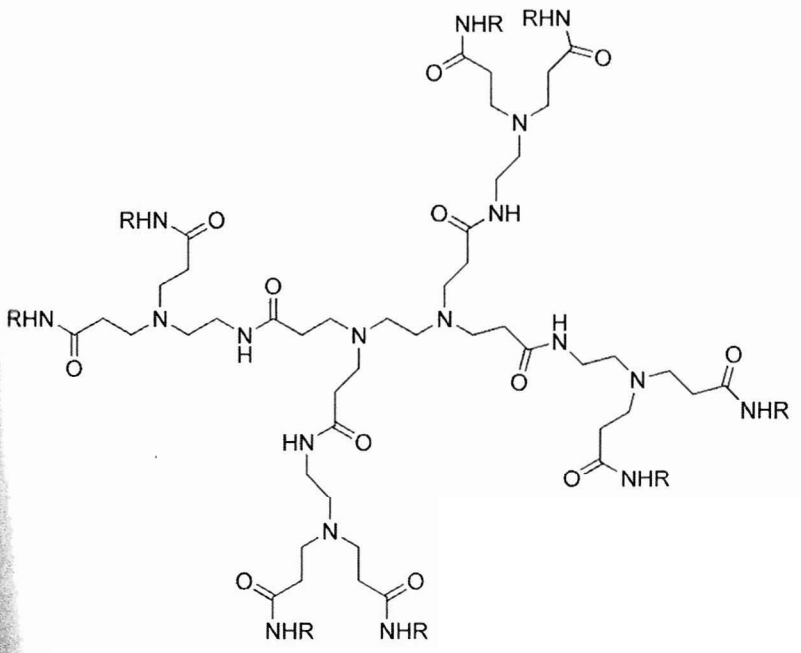

poly(acrylamide)



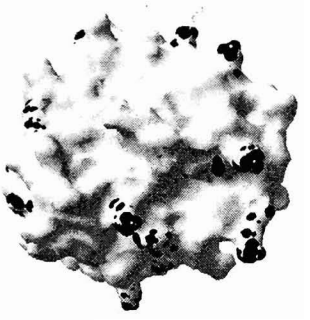

ROMP




poly(*p*-phenylene ethynylene)




PAMAM dendrimer (1st generation)



protein

**Fig. 2.10**
Representative examples of scaffolds for polyvalent ligands: polyacrylamide [6, 7, 110], ROMP [106], poly(*p*-phenylene ethynylene), PAMAM dendrimer first generation [111], and protein [109, 112]. The protein was rendered as a molecular surface (shaded gray) with displayed ligands shown in black using Swiss PDB Viewer and deposited atomic coordinates (PDB 1V9I) [113]. A more expansive collection of scaffolds for oligovalent ligands can be found in the book by Choi [16].

branched polymers have the advantage that they may be better able to interact with a number of cell surface receptors than can dendrimers. Dendrimers have the advantage that they have a relatively small influence on the viscosity of a solution, while extended, linear polymers can make solutions intractably viscous. The flexibility of polymers can be varied by the selection of the backbone: flexible (e.g., polyacrylamide) [2, 4–10, 14, 15, 90, 106, 107] or rigid [e.g., poly(*p*-phenylene ethynylene)] [108]. Rigid polymers often have poor solubility in water, and are often not truly rigid [101]. We have reported the use of proteins as scaffolds for monodisperse polymers: lysine residues of proteins were perfunctionalized with ligands [109]. These scaffolds can be either roughly spherical (if the modified protein is allowed to re-fold into its native state) or extended (if analysis is conducted under denaturing conditions, in non-aqueous solvents, or if the native structure of the protein is so significantly perturbed that it cannot re-fold).

### 2.6.3 Choice of Linker for Multivalent Ligands

Figure 2.9c shows some common linkers that have been used in the literature to connect the ligands to the scaffold for multivalent ligands [16]; the linkers are arranged approximately in order of decreasing flexibility. We proceed to discuss the advantages and disadvantages of using rigid (Section 2.6.3.1) and flexible (Section 2.6.3.2) linkers in the design of multivalent ligands.

#### 2.6.3.1 Rigid Linkers Represent a Simple Approach to Optimize Affinity

The simplest theory (Section 2.4.5) suggests using rigid linkers (those at the bottom of Fig. 2.9c) for multivalent ligands to optimize avidity for multivalent receptors. Such linkers (Fig. 2.11a) minimize the loss in conformational entropy upon complexation, but maximize the risk of unfavorable interactions between ligands, linkers, and receptors. The loss in conformational entropy reflects the restriction of modes of motion of the linker upon complexation (Fig. 2.7). The length of the linker should place the ligands at a distance that approximately matches the dis-

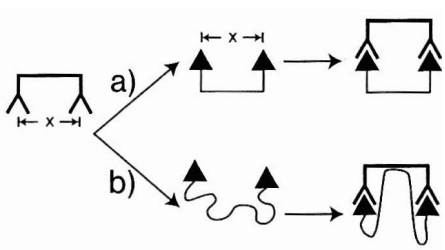



**Fig. 2.11**
Schematic representation of two different approaches to linker design for oligovalent systems: (a) a rigid linker whose length exactly matches the spacing of the receptor binding sites, (b) a flexible linker that interacts favorably with the surface of the receptor upon binding.

tance between binding sites of the multivalent receptor. This approach requires, of course, extensive experimental study into the system (e.g., spacing and geometry of binding sites, etc.); computational study may also be useful, although it has not so far been helpful. Otherwise, a poor fit (e.g., destabilizing, non-bonded interactions between the linker and the receptor, less-than-ideal interaction between each receptor and ligand due to sub-optimal positioning by linker) will result and yield a protein–ligand complex with low stability.

#### 2.6.3.2 Flexible Linkers Represent an Alternative Approach to Rigid Linkers to Optimize Affinity

An alternative approach to using rigid linkers is to use flexible linkers in the design of multivalent ligands. A flexible linker can adopt a number of conformations without steric strain (unlike rigid linkers) and allow the multivalent ligand to sample conformational space to optimize the binding of the tethered ligands to multiple receptors. This sampling of conformational space reduces the possibility of a sterically obstructed fit, a circumstance that can occur readily with rigid linkers. The flexible linker should be longer than the spacing between receptors in the multivalent receptor target to allow this sampling of conformational space; but "how much longer is optimum?" is not a question that has, so far, been answered. This approach can, theoretically, generate a design that suffers from a significant loss in conformational entropy on binding (by requiring a defined conformational state for a number of rotors upon complexation; Fig. 2.7) and thus an increase in $K_d^{avidity}$. If the linker is able to interact favorably with the oligovalent receptor, favorable enthalpic contacts [$\Delta H^{\circ}_{linker} < 0$, Eq. (3)] might make association less unfavorable than expected based on this model (Fig. 2.11 b). Alternatively, a model based on effective concentration (Fig. 2.6) predicts a much smaller loss in conformational entropy upon complexation for long, flexible linkers than the model based on the assumption of bonds that are free rotors, which become completely restricted upon oligovalent receptor–ligand association. This $C_{eff}$ model has been used to rationalize the tight-binding (low values of $K_d^{avidity}$) observed for oligovalent sugars tethered by flexible linkers binding to pentavalent toxins (Section 2.5.2.3) [38, 105].

### 2.6.4 Strategy for the Synthesis of Multivalent Ligands

The synthesis of oligovalent ligands (the connection of the individual ligands to the linkers and the scaffold) generally follows established methods of small-molecule organic synthesis and will not be discussed here. We also do not discuss the synthesis of polyvalent ligands such as dendrimers, because their synthesis has been discussed in detail elsewhere [114–116]. We focus instead on polymers (the most common types of polyvalent ligands). There are two general approaches to the synthesis of polyvalent polymers: polymerization of ligand monomers (Section 2.6.4.1), and reaction of ligands with a pre-formed activated polymer (Section 2.6.4.2).

#### 2.6.4.1 Polyvalent Ligands: Polymerization of Ligand Monomers

Kiessling and co-workers polymerized ligand-functionalized monomers directly, using ring-opening metathesis polymerization (ROMP) [117, 118]; this process yields fully functionalized polymers of controllable valencies and lengths (Fig. 2.10) [13, 106, 112, 119–121]. Polymers with less than quantitative loading of ligand have been synthesized by co-polymerizing ligand-functionalized and unfunctionalized monomers [6, 110]. The loading of the polymer with ligand depends on the ratio of ligand-functionalized to unfunctionalized monomer and their relative reactivities [122]. This approach of polymerizing ligand-functionalized monomers offers the following advantages: (i) monomers can be fully characterized before polymerization, and (ii) controllable valencies are accessible using ROMP. There are several disadvantages: (i) the need for polymerization techniques that are compatible with the functional groups on the ligand, (ii) the difficulty in synthesizing ligand-functionalized monomers, (iii) the difficulty in predicting the loading density of ligands (when ligand-functionalized and unfunctionalized monomers are co-polymerized and have a difference in reactivity towards polymerization), and (iv) the difficulty in determining the distribution of ligands (when ligand-functionalized and unfunctionalized monomers are co-polymerized) along the polymer backbone (block co-polymers or random co-polymers often cannot be readily distinguished).

#### 2.6.4.2 Polyvalent Ligands: Functionalization with Ligands after Polymerization

In the other common synthetic route to polyvalent polymers, monomers with reactive groups (e.g., activated carboxylic acids) have been synthesized and then polymerized [7, 107, 123]. In a subsequent step, the activated polymer is allowed to react with the desired ligands. The loading is controlled by the amount of ligand that is allowed to react with the polymer (reaction yields are often nearly quantitative [7]). This approach offers many advantages: (i) large amounts of activated polymer can be synthesized and then coupled with different amounts of ligand (allowing screening of the influence of ligand density of the polymer on biological activity), (ii) ligands with diverse functionality can be used because the ligands are introduced after polymerization, (iii) these ligands are often easier to synthesize than ligands activated for polymerization, (iv) synthesis of multifunctional polymers (containing two or more different functionalities) is straightforward, and (v) constant length and polydispersity of polymers are ensured if the same batch of activated polymer is coupled to different types or amounts of ligands. The constant polymer backbone removes a complicating variable from determining the effect of different types and densities of ligand(s) on biological activity. The approach has the following disadvantages: (i) it can be challenging to obtain fully functionalized polymer, (ii) the activated polymer (e.g., N-hydroxysuccinimidyl-containing polymer) is often susceptible to hydrolysis or other side reactions, and (iii) determining the composition of a polymer after functionalization with ligand can be challenging ($^1$H NMR can be used, but the resonances are usually quite broad. When the ligand contains chromophores, UV-Vis offers a more quantitative alternative to NMR) [15, 42].

## 2.7
## Extensions of Multivalency to Lead Discovery

### 2.7.1
### Hetero-oligovalency Is a Broadly Applicable Concept in Ligand Design

All of the theory regarding homo-bivalency applies to hetero-bivalent systems, with appropriate incorporation of multiple dissociation constants for the monovalent interactions and statistical factors. Further, hetero-bivalency is expected to be more broadly applicable than homo-bivalency, since it is applicable to problems requiring the ability to target monomeric proteins, which do not contain multiple, proximate, identical binding sites, but may have "sticky" sites adjacent to binding sites.

The examples presented here are merely meant to be representative of the application of hetero-bivalent ligands to target monomeric proteins (proteins which do not have two identical binding sites). The additional site can be either a hydrophobic patch on the protein or a binding site for a second substrate [124]. Using a model protein for ligand design – carbonic anhydrase II (CA) – we demonstrated this targeting of a secondary site (a hydrophobic patch) near the active site of an enzyme [96, 125, 126]. This patch was relatively non-discriminating towards the type of hydrophobic group in the ligand, depending only on the size of the group: for instance, ligands with benzyl, adamantyl, and octyl groups all bound with roughly the same (~ nM) avidity [96]. Finn, Sharpless, and co-workers applied a Huisgen 1,3-dipolar cycloaddition templated by acetylcholinesterase to generate a tight-binding ligand for the enzyme ($K_d^{avidity}$ = 77–410 fM) from known monovalent ligands ($K_d^{affinity}$ ~ nM–μM) that bound two different sites on the enzyme: the active site and a "peripheral" site at the rim of the active site gorge [127]. Rosenberg, Fesik, and co-workers reported similar covalent tethering to synthesize a high-avidity ($K_d^{avidity} \leq 1$ nM) hetero-bivalent ligand for Bcl-2 family proteins from low-affinity monovalent ligands ($K_d^{affinity}$ ~ mM) to an active site and an adjacent hydrophobic patch (Chapter 9) [128]. Parang and Cole reviewed the application of hetero-bivalent ligands to protein kinases, enzymes that transfer a phosphate group from adenosine triphosphate (ATP) to protein targets [129]. These ligands usually consist of a nucleotide analog and a peptide to bind to the ATP-binding site and protein-binding site of the enzyme, respectively. Such bisubstrate inhibitors were shown to exhibit selectivity between the members of the family of protein kinases. Theravance (San Francisco, Calif.) [130] reported the synthesis [131, 132], *in vitro* antibiotic activity against conventional and antibiotic-resistant strains of bacteria [133–135], and phase II clinical trials of telavancin [136], a derivative of vancomycin containing a hydrophobic and a hydrophilic side-chain [131]. The anti-bacterial action of telavancin occurs by two mechanisms: disruption of cell wall biosynthesis (similar to vancomycin itself) in Gram-positive bacteria, and depolarization (i.e., disruption) of the bacterial cell membrane [135]. An injectable form of the compound is currently in Phase III clinical trials for the treatment of complicated skin and skin structure infections (cSSSI) and hospital-acquired pneumonia (HAP) [131]. Telavancin demonstrates the therapeutic potential of hetero-bivalent ligands.

### 2.7.2 Dendrimers Present Opportunities for Multivalent Presentation of Ligands

A number of sugar-displaying dendrimers have been evaluated for binding to lectins or the inhibition of hemagglutination induced by either bacteria or lectins. Lundquist and Toone reviewed these studies [17]. In particularly well controlled studies, Cloninger and co-workers examined the influence of PAMAM dendrimer size (generations 1–6) and loading density of mannose on Con A-induced hemagglutination of erythrocytes [111, 137, 138]. They found that, for fully mannose-functionalized dendrimers, only large dendrimers (generations 4–6 with >50 sugars per dendrimer) were able to inhibit hemagglutination with significant enhancements; they attributed this result to the large dendrimers being able to bind bivalently to Con A (the spacing between binding sites on Con A is ~6.5 nm) [137]. In a follow-up study, they examined the influence of loading density of mannose on the dendrimer on hemagglutination [111]. The enhancements (on a per *mannose* basis) for the different-sized dendrimers (generations 4–6) all peaked at ~50% of the maximal loading of mannose (maximum enhancements of 250–600, scaling with generation number, were observed). The investigators speculated that steric interactions between mannose residues decreased the enhancement at higher loadings. The enhancement (on a per *dendrimer* basis) increased monotonically with the loading density; this increase (with no peak) was attributed to statistical effects (avidity entropy).

Dendrimers are also promising as agents in human health care [115, 139]. Starpharma (Australia) [140] recently completed Phase I clinical trials on VivaGel, a topical vaginal microbicide that prevents infection by HIV *in vivo* (primate models) [141]. The active ingredient in VivaGel is a fourth generation dendrimer (SPL7013) decorated with naphthalene disulfonate groups; this polyanionic coating is believed to bind to the viral coat (presumably, via electrostatic interactions) and prevent the attachment of the virus to host T cells [142].

### 2.7.3 Bivalency in the Immune System

IgG and IgE antibodies, prime components of the immune system, are bivalent proteins containing two identical receptors (Fab sites; Fig. 2.12) [21]. When binding bivalently to a surface (Fig. 2.12a) or to a soluble bivalent ligand (Fig. 2.12b), we postulate that the enhancement (β) for a given antibody is inversely proportional to the monovalent dissociation constant ($K_d^{affinity}$) and directly proportional to the effective concentration ($C_{eff}$) of ligand near an available receptor (Fig. 2.12). If we assume $C_{eff}$ to be constant for all antibodies (that is, that they have the same average distance between Fab sites), then greater enhancements will result from higher affinity (lower $K_d^{affinity}$) ligands. At cell surfaces, the enhancement for the binding of a polyclonal mixture of IgG with high monovalent affinity (average $K_d^{affinity} \sim 1$ nM) to the surface of *Bacillus* sp. was ~100 [143]. Cremer and co-workers examined the binding of a polyclonal mixture of IgG to phospholipid mem-

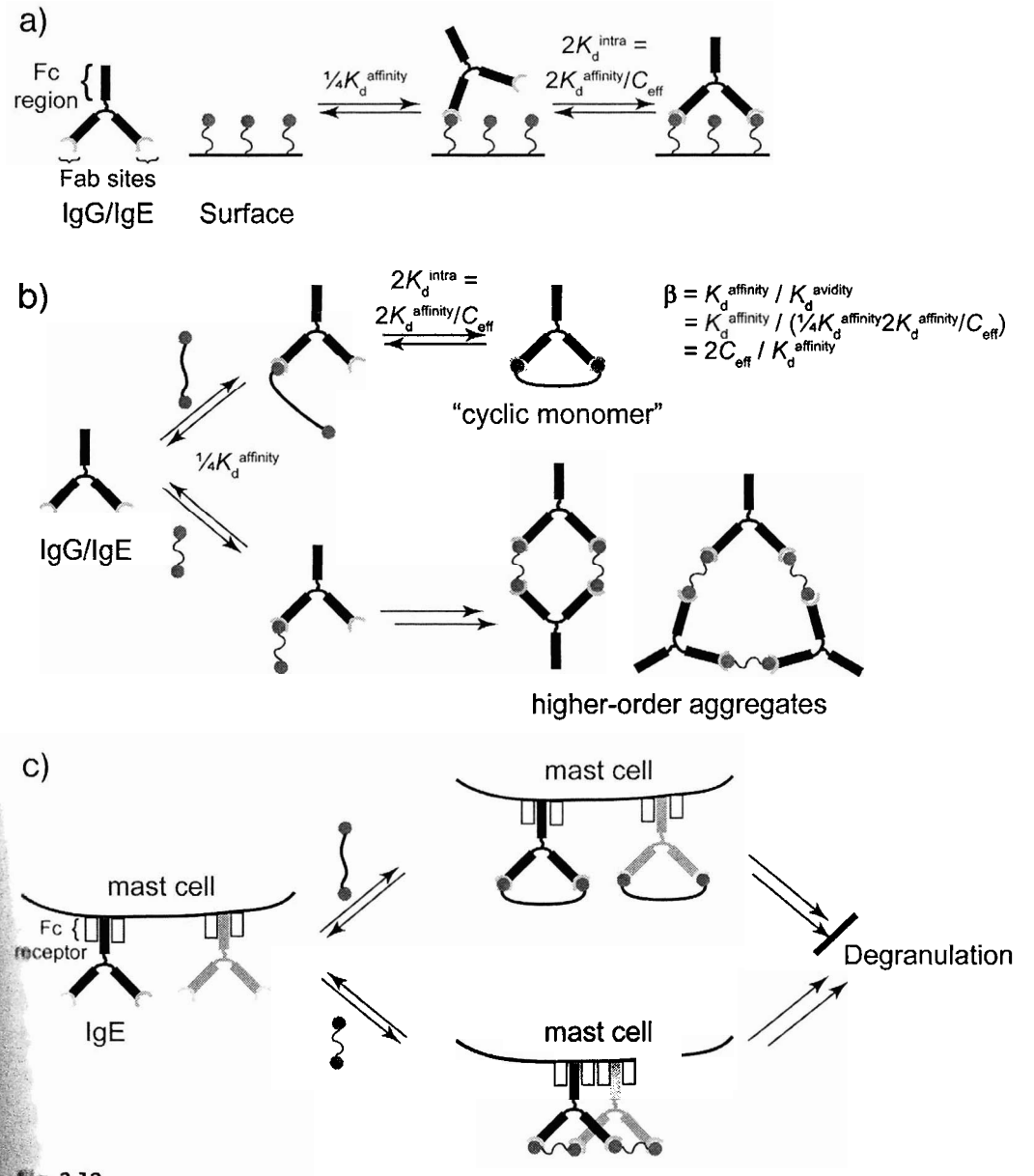



**Fig. 2.12**
Binding of bivalent antibodies (IgG or IgE) to bivalent ligands and surfaces. (a) The stepwise equilibria characterizing binding of an antibody bivalently to a surface displaying ligands are shown. The dissociation constant for the second step ($K_d^{intra}$) is taken to be the theoretical value assuming no cooperativity between Fab sites (Fig. 2.6). (b) Two different pathways are available to antibodies binding to bivalent ligands in solution depending on the length of the linker in the bivalent ligand [100, 145]. The dissociation constants for the top pathway (intramolecular ring closure) are analogous to those in (a). The enhancement (β) applies to both (a) and the top pathway in (b). (c) IgE bound to mast cells by their Fc regions can bind to bivalent ligands in two different pathways analogous to those in (b), again depending on the length of the linker in the bivalent ligand [102, 146]. These two pathways have different effects on degranulation of the mast cells: bivalent ligands with long linkers form "cyclic monomers" and inhibit degranulation, while bivalent ligands with short linkers cross-link the surface-bound IgEs and induce degranulation. The two IgEs are shaded differently to aid visualization of the aggregate.

branes containing ligand lipids; an enhancement of ~40 was observed for the weak-affinity system studied ($K_d^{affinity} \sim 50$ μM) [144].

Pecht, Licht, and co-workers used bivalent ligands with long, rigid poly(proline) linkers to examine the formation of soluble IgE "closed monomers": both receptor sites of the antibody were bound to the same bivalent ligand (Fig. 2.12b, top pathway) [100]. They measured enhancements of roughly the same order of magnitude ($\beta \sim 20$) for medium-affinity ligands ($K_d^{affinity} \sim 0.1$ μM) containing sufficiently long linkers to bridge both Fab sites as for the cell-surface results. The relatively low enhancements observed in these studies indicate low values of $C_{eff}$ of a ligand in proximity to an available (unbound) Fab site of the antibody. Consistent with this low value of $C_{eff}$, crystal structures revealed that the two receptors on an antibody are ~8 nm apart and are flexibly coupled to one another [147–149]. We expect this large distance and flexible coupling to increase the entropic cost of association (Section 2.4.5).

A number of investigators showed that bivalent ligands too short to form "closed monomers" can form discrete cyclic antibody aggregates (e.g., cyclic dimers where two antibodies are bridged by two bivalent ligands; Fig. 2.12b, bottom pathway) [104, 150–156]. In solution, these aggregates were shown to be stable on relatively long time-scales (for analysis by HPLC and ultracentrifugation) [104, 155–157].

Holowka and Baird discussed the importance of aggregation of IgE (that are bound to mast cells by their Fc region) on the release of histamine, a process referred to as degranulation (Fig. 2.12c) [158]. Degranulation of mast cells can be either inhibited or promoted by the binding of oligovalent ligands to IgE bound to mast cells: long oligovalent ligands that can form "closed monomers" (i.e., span both Fab sites of an IgE; bind *intra*molecularly to one IgE) can inhibit degranulation (Figure 2.12c, top pathway), while short oligovalent ligands that aggregate IgEs *inter*molecularly result in degranulation (Fig. 2.12c, bottom pathway). Baird and co-workers reported the inhibition of degranulation of mast cells by bivalent ligands containing long oligo(ethylene glycol) linkers ($\geq 9$ units) [146] and long DNA linkers (30-mer; bivalent ligands with shorter DNA linkers promoted degranulation) [102], and by large ligand-displaying dendrimers (smaller dendrimers promoted degranulation) [146]. They observed enhancements (β) of up to 100. Ligands that inhibit the degranulation of mast cells could be useful in the treatment of allergies.

### 2.7.4
### Polymers Could Be the Most Broadly Applicable Multivalent Ligands

We demonstrated that polymers displaying two types of functionalities (bifunctional polymers) can bind to and display a functional element on the surface of synthetic surfaces (SAMs) and bacterial surfaces [15]. In this proof-of-principle demonstration, the polymer-labeled surface was efficiently bound by antibodies (directed against the functional element); this binding shows that a new functionality can be *introduced* onto a surface by using bifunctional polymers. This approach

should be general: as long as a recognition element (ligand) with some affinity ($K_d^{affinity}$ ~ mM–μM) can be found, a tunable functional element (e.g., fluorophore, hapten) can be incorporated onto any surface.

There are a number of conventional methods to discover ligands of moderate affinity for target receptors (e.g., combinatorial chemistry [159, 160], phage display [161], *de novo* design [92]). Our work designing polymers that were effective against anthrax toxin demonstrated the discovery of a low-affinity ligand and the conversion of this ligand to a high-avidity inhibitor using polyvalency [42]. A peptide that inhibited the assembly of the components of anthrax toxin with low affinity ($IC_{50}^{affinity}$ = 150 μM) was discovered by phage display and was converted by polyvalency into a high-avidity polymer ($IC_{50}^{avidity}$ = 20 nM on a per-peptide basis). This polymer was effective in protecting an animal model against challenge with purified components of anthrax toxin [42].

Kiessling and co-workers demonstrated that the enhancement of sugar-displaying polymers in the inhibition of binding of model leukocytes to artificial surfaces is greater in dynamic, cell-rolling assays (Section 2.5.1; β = 170 on a per sugar basis) than in static, cell-free assays (β = 5 on a per sugar basis) [2]. Since the dynamic assays are more physiologically relevant than the static ones, these results suggest that this polymer could be more effective *in vivo* than predicted by static assays. Generalizing this single result to the potential *in vivo* efficacy of other polymers is, of course, not possible. Kiessling and co-workers also investigated the clustering of cell-surface receptors by multivalent polymers displaying galactose and the influence of this clustering on such downstream effects as bacterial chemotaxis [11–13, 162].

Copaxone (glatiramer acetate), marketed by Teva Pharmaceuticals (Israel), is an example of a highly successful polymer therapeutic [163]. Glatiramer acetate is a large (average MW = 5–11 kDa) synthetic polypeptide of L-Ala, L-Glu, L-Lys, and L-Tyr (a mimic of myelin basic protein) that is random in distribution but defined in composition [164]. It has been approved for the treatment of patients with relapsing-remitting multiple sclerosis (RR-MS; reducing the frequency of relapses and disease progression in clinical studies) [165]. While the exact mechanism of action is unknown, the polypeptide is known to attenuate patients' autoimmune response to myelin and to reduce both the inflammation and neurodegeneration associated with the disease [164]. An oral form of glatiramer acetate is effective in the treatment of models of MS *in vivo* (rodents and primates) [166, 167]. It has not yet, however, shown statistically significant results in human clinical trials (as of 2005, Phase II clinical trials were ongoing) [163]. The oral form of the drug has been shown to be non-toxic, providing hope that perhaps higher doses will be effective at treating the disease in humans [167, 168].

## 2.8
## Challenges and Unsolved Problems in Multivalency

A number of unanswered questions about multivalency still remain: Why do we not achieve the theoretical maximum of enhancement in any multivalent system [even with seemingly well designed systems such as trivalent D-Ala-D-Ala/vancomycin (Section 2.5.2.1; Fig. 2.4)]? Why does the experimentally observed avidity not scale with valency ($i$; Fig. 2.4)? What principles of design will allow one to approach this theoretical maximum?

What is the best linker for a multivalent ligand (in terms of length, flexibility, chemical composition)? What is the mechanism of polyvalent binding to surfaces (e.g., polymers and dendrimers)? How do we design the most effective multivalent ligands? How can this effectiveness be demonstrated (e.g., what kinds of assays?) in a way that attracts the active interest of the pharmaceutical industry?

How should multivalent ligands (primarily polyvalent ones) be manufactured for therapeutic use? Will the innate polydispersity of polymers pose exceptional problems in regulatory clearance [169]?

## 2.9
## Conclusions

Multivalency can convert weak monovalent ligands into oligovalent ligands effective at low concentrations. The discovery of low-affinity monovalent ligands ($K_d^{affinity} \sim$ mM–μM) is less challenging than that of high-affinity monovalent ligands ($K_d^{affinity} \sim$ nM). These weak monovalent ligands can be discovered through rational design or through screening efforts (e.g., combinatorial chemistry [159, 160], phage display [42, 161]). For a hetero-bivalent inhibitor, the second binding site can be as non-specific as a hydrophobic patch on a monomeric protein, or can involve an additional substrate binding site. Polyvalent ligands present new mechanisms of action that are not available to monovalent ligands. Examples include steric inhibition of binding to a surface [4, 5, 7, 8], shielding one set of receptors on a surface by binding to another set, incorporating a new functional element onto a cell surface (e.g., "painting" a bacterial surface with antigens) [14, 15, 170], "cloaking" the antigenicity of a surface, and forming structured aggregates of antibodies and other species.

The design principles and mechanism of action of multivalency are still not entirely clear. For instance, Kitov and Bundle designed a decavalent ligand to bind 1:1 with the pentavalent Shiga-like toxin (to take advantage of two of the three receptor sites per monomer) [32]. They discovered, surprisingly, that a 2:1 complex of toxin to inhibitor was favored (Section 2.5.2.3). This example illustrates some of the limitations of our understanding of multivalent ligand design.

Multivalent ligands are generally larger (higher molecular weight) than monovalent ones. This greater size can decrease bioavailability (especially oral bioavailability), decrease rates of excretion, and limit tissue permeation. Multivalent

ligands are typically more difficult to synthesize and characterize than monovalent ones. Polymers (the most common polyvalent ligands) are not single chemical entities and require special techniques for reproducible synthesis and manufacture. Multivalent species (especially polymeric polyvalent species) are unfamiliar to the FDA, and from the point of regulation will require new rules and regulations before approval [169].

There are a number of potential therapeutic applications of multivalent ligands. Hetero-bivalent ligands can, in principle, be imagined for most proteins (exploiting a secondary site). The immune system relies fundamentally upon bivalency; to interact effectively with components of it (e.g., antibodies) requires a multivalent approach (Section 2.7.3). Bifunctional polymers are able to introduce a new functional element to cell surfaces [170]. Theoretically, any class of cells (e.g., pathogens, cancer cells) can be targeted by this approach, the only requirement being a ligand–receptor interaction of modest affinity ($K_d^{affinity} \sim$ mM–μM) and a reasonable surface density of receptors. We believe that polymers will be particularly effective in places where their large size is an advantage rather than a disadvantage. Examples could include administration to the digestive tract, respiratory system, eye, superficial wounds, and vagina, where retaining the polyvalent ligand in that organ or structure is useful, and where release into the systemic circulation is undesirable.

## Acknowledgments

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