SYNTHETIC MULTIVALENT MOLECULES

Concepts and Biomedical Applications

SEOK-KI CHOI

A JOHN WILEY & SONS, INC., PUBLICATION

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PREFACE

This book presents basic and advanced principles underlying the multivalent interactions that are prevalent in biological systems. To illustrate important or complex concepts, the book provides up-to-date examples of synthetic multivalent molecules, their design, and their biological benefits. Functional roles displayed by such molecules of both natural and synthetic origin are well documented in biology, where they exert unique and crucial activities at a level not readily achievable by monovalent molecules. The concept of multivalent design is now accepted as an effective strategy—in particular, for designing ligands, inhibitors, and drugs that influence biological systems potently and selectively.

Over the past 15 years, diverse diciplines have generated a growing interest in the biomedical application of multivalent design. The goal of this book is to share the findings in this exciting area of research by providing a systematic summary of experimentally tested case studies of multivalency. I believe that a single book serves best to collect such scattered research material in one place and to discuss it in a consistent and introductory format.

The book focuses on practical examples of synthetic multivalent molecules reported broadly in the literature. It consists of five chapters. In Chapter 1 I introduce the multivalent molecule and its structural elements, describe the mechanistic basis that accounts for the benefits of multivalent interaction, and provide a short summary of biological functions displayed by multivalent molecules. In Chapters 2 to 4 I provide practical examples derived from biological targets in viral, bacterial, and mammalian cells, respectively. In each chapter I review in a similar format the design concept, synthesis, and biological activity of multivalent molecules: in particular, those of synthetic origin.

Typically, the chapter format begins with a brief description of a target from a structural and functional viewpoint to provide a rationale for multivalent design, followed by the main discussion. In Chapter 5 I summarize various aspects of synthetic methods used in the synthesis of multivalent molecules, and I conclude with a summary of combinatorial approaches developed in the library design of multivalent molecules. The book ends with an appendix that presents tabular summaries of both examples treated in this book and untreated. The targets selected for discussion are comprehensive but, of course, do not purport to be a complete list.

The book is written for a broad community of audiences, comprising educators, graduate students, and professional researchers in academia and the (bio)pharmaceutical industry, particularly those who perform interdisciplinary research in organic chemistry, chemical biology, biological chemistry, medicinal chemistry, pharmacology, and medicine. Specifically, it would be most valuable as a reference book for those scientists interested in finding new ideas and developments in areas of receptor–ligand interaction, carbohydrate-based medicines, enzyme inhibitors, toxin inhibitors, DNA(RNA)–drug association, antibiotics, antiviral agents, anti-inflammatory drugs, and anticancer therapeutics.

It is my pleasure to express great gratitude to Professor Koji Nakanishi of Columbia University, who introduced me to the importance of multidisciplinary bioorganic studies and encouraged this publication. I am also indebted to Professor George M. Whitesides of Harvard University and sincerely appreciate his directing me to continue research in multivalency. In 1996, Professor Whitesides and several colleagues, including Professor John Griffin of Stanford University, Dr. Mathai Mammen of Harvard, and James Tananbaum of Sierra Ventures, founded Advanced Medicine, Inc. to develop opportunities in multivalent drug research and development. I joined Advanced Medicine in 1997 to help further develop ideas in multivalent drug design. Today, Advanced Medicine has evolved into a vibrant pharmaceutical company, Theravance, Inc., under the guidance of our Chairman, Roy Vagelos (former CEO of Merck Pharmaceuticals), and current CEO, Rick Winningham. I am indebted to my colleagues at Theravance for ongoing collaborations in medicinal chemistry. In particular, I am sincerely thankful to Dr. Ed Moran, Dr. Thomas Jenkins, and Dr. Mathai Mammen for their editorial assistance with this manuscript.

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Palo Alto, California SEOK-KI CHOI

NOTES FOR ORGANIZATION AND CLASSIFICATION

Given diverse aspects of classification related to multivalent ligands and inhibitors, I wish to provide a road map to the structure and organization of this book. Its main contents consist of three chapters that are divided according to the origin of targets: viruses (2), bacteria (3), or mammalian cells (4). Within this division, materials are grouped further on the basis of target class and structural composition of multivalent molecules. Therefore, typical headings of each chapter start with an organism (e.g., influenza virus), proceed to a description of a target under a certain class (receptor: hemagglutinin), followed by a discussion of a group of multivalent molecules (e.g., divalent, trivalent, polyvalent, . . . , sialic acid). Under such a format, multivalent ligands from the same class can be reviewed collectively and comparatively as to their design concept, synthesis, and biological activity. A short outline of each chapter is given below.

Chapter 2 covers multivalent examples from viral targets such as influenza virus and the human immunodeficiency virus (HIV). Several classes of multivalent inhibitors targeting viral surface proteins (influenza hemagglutinin), enzymes (influenza neuraminidase, HIV-protease), and nucleic acids (as intercalators and as binders at minor or minor groove) are presented. The chapter ends with a summary of synthetic multivalent antigens that are designed to mimic the surface of influenza, HIV, or foot-and-mouth disease virus.

Chapter 3 focuses on bacterial cells, a pathogenic system that proves to be well validated for a multivalent approach. This chapter provides ample examples of multivalent inhibitors that display enhanced activity against a wide range of receptors or ligands expressed on a bacterial surface (D-ala-D-ala, lipid A, adhesins), toxins (cholera, anthrax), enzymes (transpeptidase), and nucleic acids (ribosomal RNA).

Chapter 4 treats multivalent molecules that inhibit or modulate mammalian cells. The list of tested macromolecular targets is extensive. Selected examples include surface receptors (hepatic asialoglycoprotein receptor, selectins), enzymes (acetylcholine esterase), G-protein-coupled receptors (opioid receptor), and ion channels (cyclicnucleotide-gated channel). Applications in nucleic acid are demonstrated by a daunorubicin dimer acting as a DNA intercalator, and a hairpin-shaped polyamide dimer acting as a minor groove binder. In addition, several types of synthetic vaccines are illustrated, including multivalent globo-H presented on protein carrier as the one mimicking cancer cell surface. Discussion of such diverse cellular targets should help to better understand multivalent concepts and applications in drug development.

Finally, the organization of the book aims for easy access to and concise comparison of multivalent molecules for a given receptor. However, readers might be interested in looking at the features of multivalent molecules organized from different aspects regardless of their target families, functions, or cellular origins. In a sense, it might be useful to compare multivalent design methods within a broadly defined boundary based on target location (membrane-bound versus soluble) and target valency (divalent, trivalent, ..., multivalent). The nature of assembly of multivalent sites is considered to be important as well, such that targeting to a single-subunit multivalent receptor can be distinguished from a multisubunit receptor complex composed of either identical subunits (homo) or different subunits (hetero). To complement such interesting features, a table (Table 20) is given in the appendix as a cross-reference to present such information for major targets: for example, whether they are membrane-bound, soluble, homo-trivalent, or hetero-divalent. Readers are advised to utilize these additional aspects of classification when selecting materials.

ABBREVIATIONS

ACE	affinity capillary electrophoresis
AChE	acetylcholine esterase
Ala	alanine
AmB	amphotericin B
β-AR	β-adrenergic receptor
ASGP-R	asialoglycoprotein receptor
AT-III	antithrombin III
AZT	azidothymidine
BChE	butyrylcholinesterase
BSA	bovine serum albumin
bZIP	basic leucine zipper protein
CA-II	carbonic anhydrase II
Caspase-3	cysteine aspartyl protease-3
CD	cyclodextrin
$C_{\mathbf{eff}}$	effective local concentration
cGMP	cyclic guanosine monophosphate
CID	chemical inducer of dimerization
CNG	cyclic nucleotide-gated (CNG)
Con A	concanavalin A
CPMV	cowpea mosaic virus
CRD	carbohydrate-recognition domain
CsA	cyclosporin A

CTB	cholera toxin B subunit
CVN	cyanovirin-N
DC-SIGN	dendritic cell–specific ICAM-3 grabbing nonintegrin
DHFR	dihydrofolate reductase
DLS	dynamic light scattering
EC	effective concentration
EGFR	epidermal growth factor receptor
ELAM-1	endothelial leukocyte adhesion molecule-1
ELISA	enzyme-linked immunosorbent assay
EPO-R	erythropoietin receptor
ERK	extracellular regulated kinase
FGF	fibroblast growth factor
FKBP	FK506-binding protein
FMDV	foot-and-mouth disease virus
FRAP	FKBP-12-rapamycin-associated protein
Gal	galactose
Gal cer	galactosylceramide
GalNAc	*N*-acetylgalactoside
GalTase	galactosyltransferase
G-CSF	granulocyte-colony-stimulating factor
GGBP	glucose–galactose binding protein
Glc	glucose
Glc cer	glucosylceramide
GlcNAc	*N*-acetylglucosamine
GlyCAM-1	glycosylated cell adhesion molecule-1
gp120	glycoprotein 120
GPCR	G-protein-coupled receptor
GPI	glycosylphosphatidylinositol
HA	hemagglutinin
HAI	hemagglutination inhibition
HIV	human immunodeficiency virus
HLE	human leukocyte elastase
HMPA	*N*-(2-hydroxypropyl)polymetharylamide
HPPK	6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase
HT	5-hydroxytryptamine or serotonin
IC_{50}	concentration at 50% inhibition
ICAM-3	intracellular adhesion molecule-3
IgG	immunoglobulin G

IgM	immunoglobulin M
IL-5	interleukin-5
IP_3	inositol 1,4,5-triphosphate
ITC	isothermal titration calorimetry
JCP	jelly coat glycoprotein
K_a	association constant
K_d	dissociation constant
K_i	inhibition constant
K_i^{HAI}	hemagglutination inhibition constant
K_m	Michaelis constant
k_{off}	rate of dissociation
k_{on}	rate of association
KLH	keyhole limpet hemocyanin
Lac cer	lactosylceramide
L–B	Langmuir–Blodgett
LDL	low-density lipoprotein
Le^b	Lewis b
Le^x	Lewis x
LPS	lipopolysaccharide
mAChR	muscarinic acetylcholine receptor
Man	mannose
MBP	mannose-binding protein
MDR	multidrug resistance
MHC	major histocompatibility complex receptor
MIC	minimal inhibition concentration
MMP	matrix metalloproteinase
α-MSH	α-melanocyte-stimulating hormone
MTX	methotrexate
NA	neuraminidase
nACh-R	nicotinic acetylcholine receptor
NAD	nicotine adenine dinucleotide
NeuAc	*N*-acetylneuraminic acid (sialic acid)
NNRTI	non-nucleoside-based reverse transcriptase inhibitor
NRTI	nucleoside-based reverse transcriptase inhibitor
NT-3	neurotrophin 3
PA	protective antigen
pA	poly(acrylamide)
pAA	poly(acrylic acid)

PAMAM	poly(amide amine)
PBP	penicillin-binding protein
PEG	poly(ethylene glycol)
PKC	protein kinase C
PNA	peptide nucleic acid
pNAS	poly(*N*-acryloyloxysuccinimide)
PSA	poly-α2,8-sialic acid
PSGL-1	P-selectin glycoprotein ligand-1
PTPase	protein tyrosine phosphatase
RCA	*Ricinus communis* agglutinin
RGD	Arg-Gly-Asp
RNase S	ribonuclease S
ROMP	ring-opening metathesis polymerization
RRE	rev-response element
RSV	respiratory syncytial virus
RT	reverse transcriptase
SA	sialic acid
SAM	self-assembled monolayer
SIV	simian immunodeficiency virus
SLe^a	sialyl Lewis a
SLe^x	sialyl Lewis x
SLT	Shigalike toxin
SNP	single-nucleotide polymorphism
SPR	surface plasma resonance
SSRI	selective serotonin reuptake inhibitor
TAR	transactivator-response RNA element
VHR	vaccinia VH1–related phosphatase
VP	viral protein
VRE	vancomycin-resistant enterococci
VSE	vancomycin-susceptible enterococci
WGA	wheat germ agglutinin
ZP	zona pellulcida

1

INTRODUCTION

1.1 NOMENCLATURE AND DEFINITIONS

1.1.1 Valency

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

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

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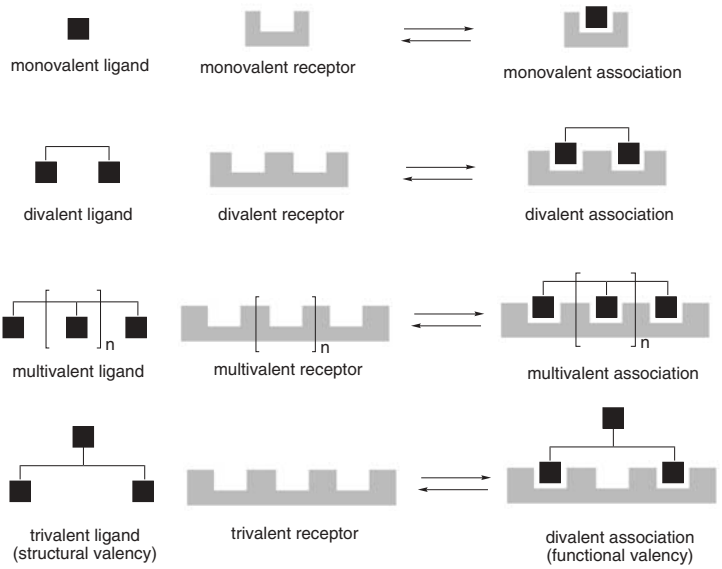

Figure 1.1 Definitions of structural and functional valence as illustrated in monovalent, divalent, and multivalent (polyvalent) interaction.

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

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

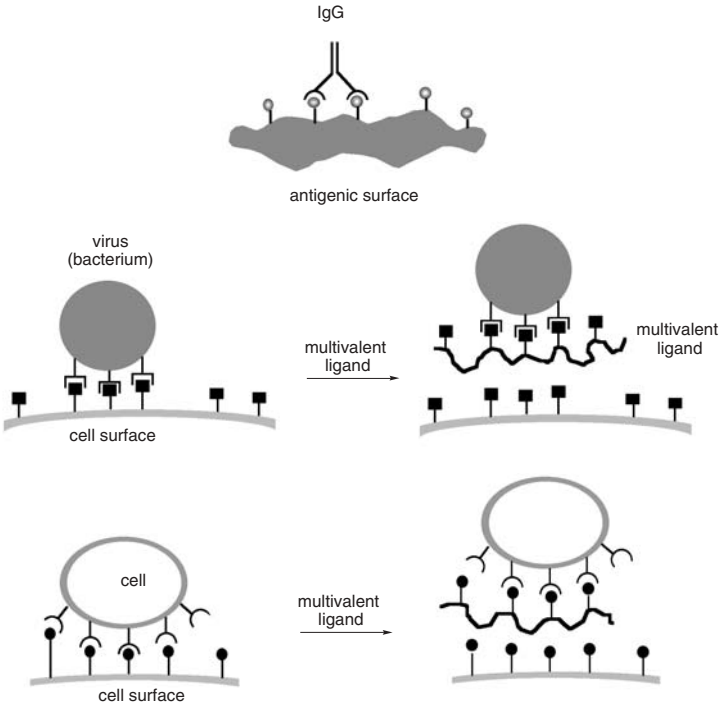

Figure 1.2 Representative examples of natural multivalent interactions and their synthetic multivalent inhibitors.

1.1.2 Linkers

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

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

1.1.3 Scaffolds

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

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

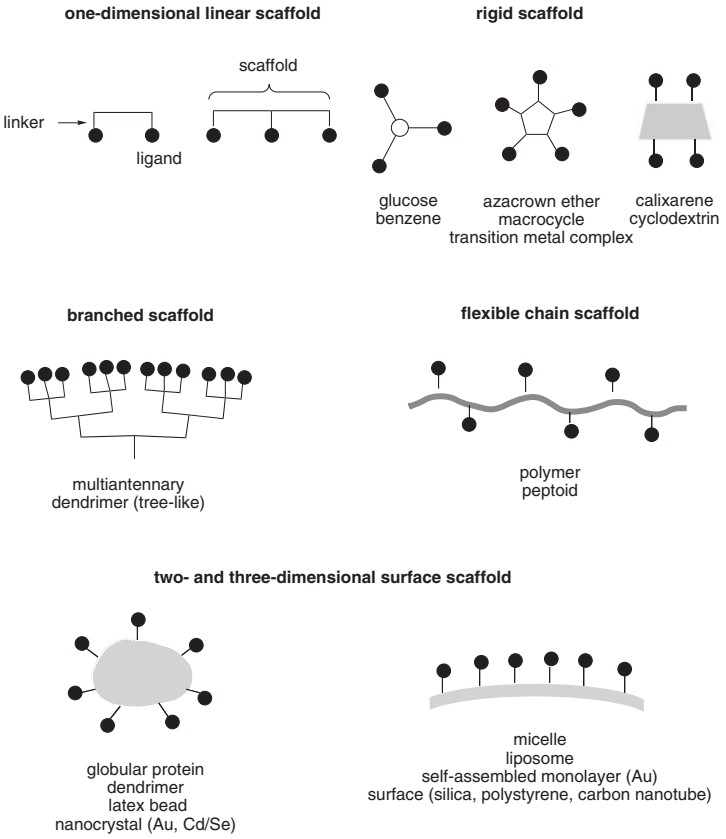

Figure 1.3 Shape and size of various scaffolds used in multivalent presentation.

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

The surfaces of naturally occurring glycoproteins present multiple types of

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

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

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

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

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

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

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

1.1.4 Ligand Density

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

1.1.5 Homo- and Heterovalent Molecules

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

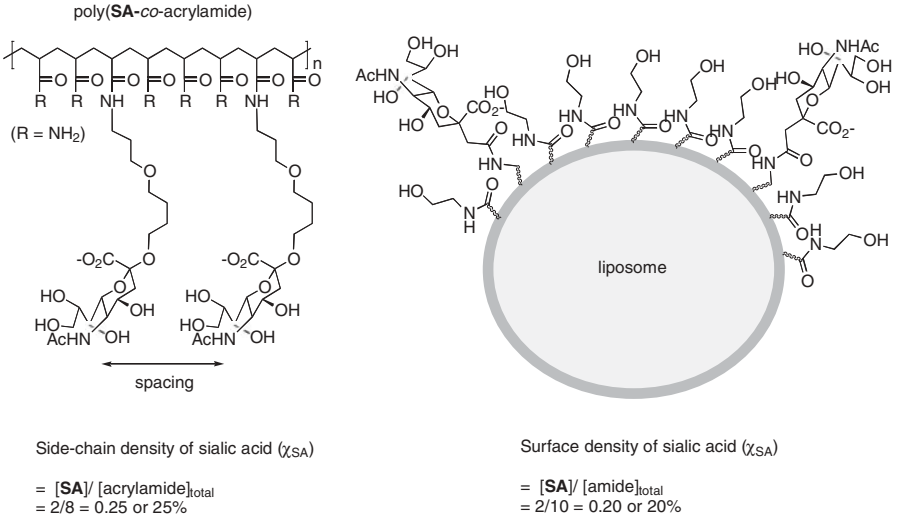

Figure 1.4 Definition of ligand density in polymer- and liposome-based multivalent molecules.

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

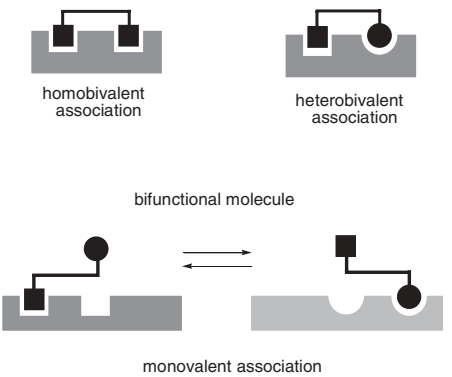

Figure 1.5 Representation of homovalent and heterovalent interaction.

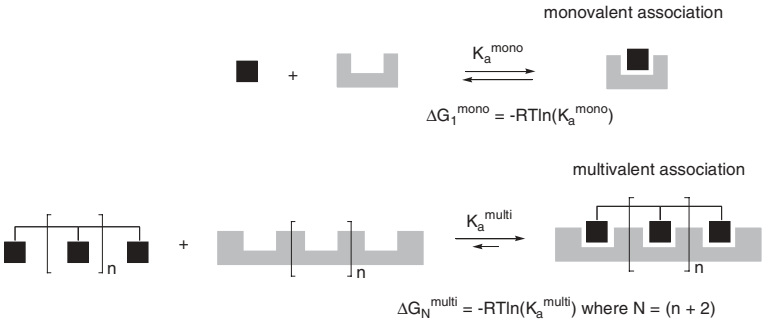

Figure 1.6 Comparison of thermodynamic parameters of association in monovalent and multivalent interaction.

1.2 MECHANISTIC ASPECTS OF MULTIVALENT INTERACTION

1.2.1 Affinity Constant and Avidity

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

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

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

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

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

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

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

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

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

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

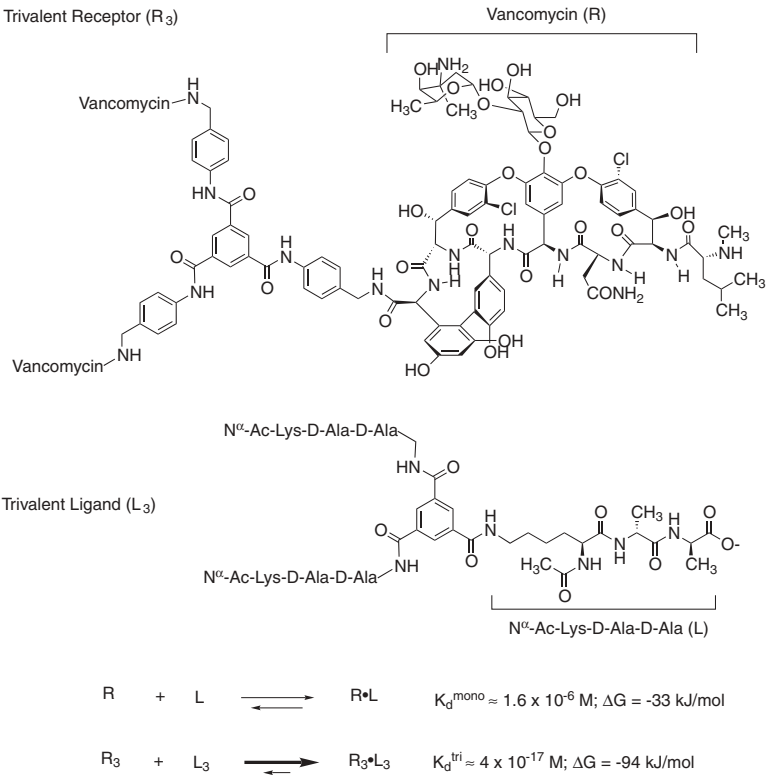

Figure 1.7 Interaction of a trivalent vancomycin with a trivalent D-Ala-D-Ala ligand.

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

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

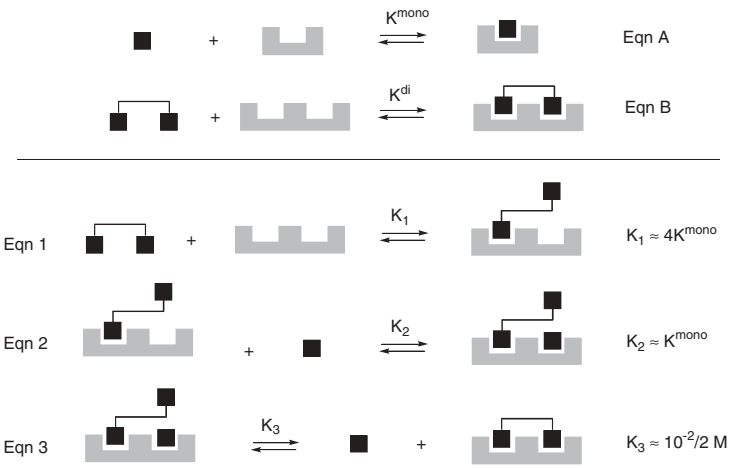

Figure 1.8 Theoretical model used for deriving an equation that would make it possible to estimate di(multi)valent association constant.

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

$$K^{\mathrm{di}} = K_1K_2K_3 = 2 \times 10^{-2}(K^{\mathrm{mono}})^2$$

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

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

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

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

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

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

1.2.2 Thermodynamics

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