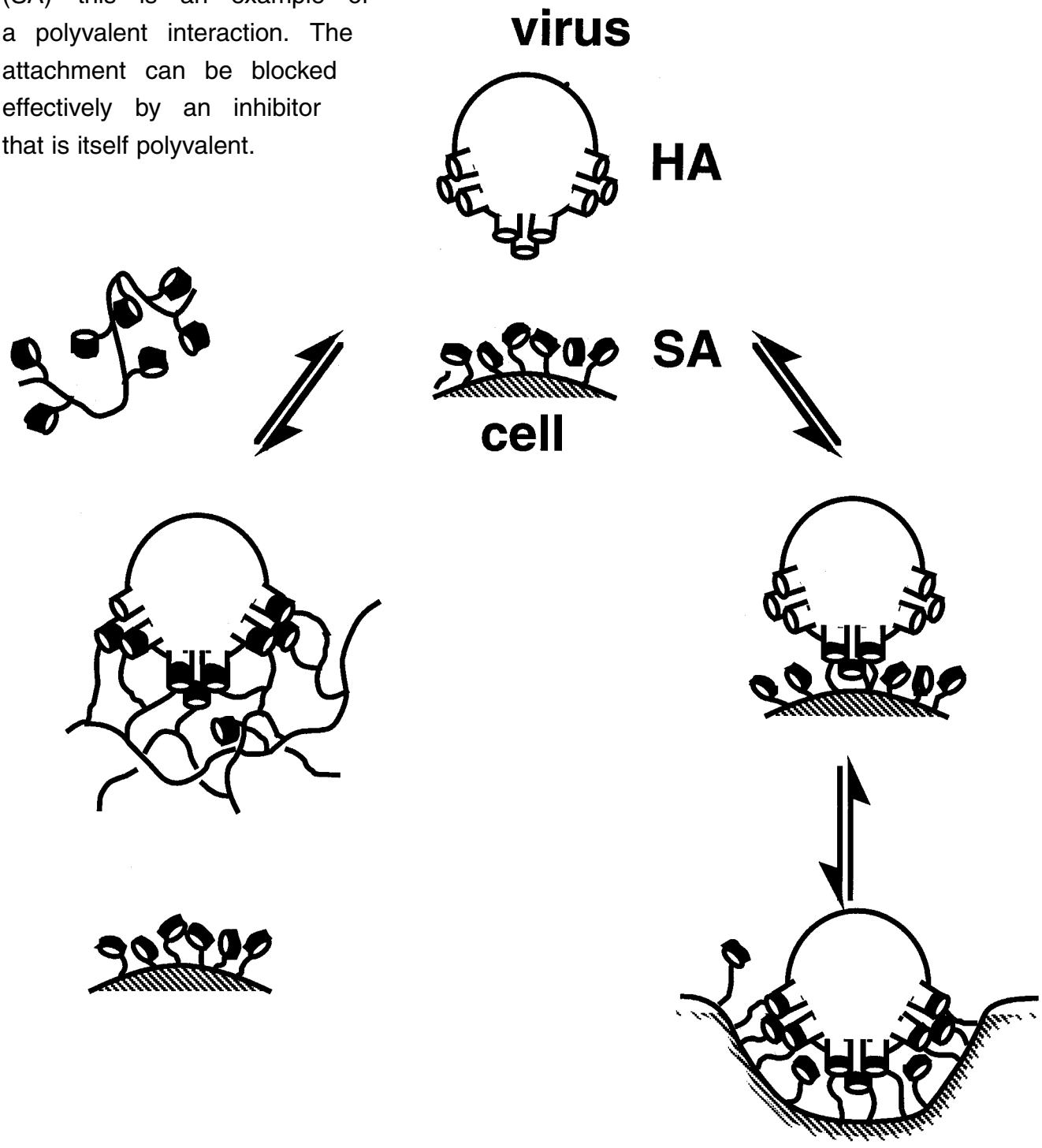
The attachment of an influenza virus to a target cell occurs through multiple simultaneous interactions between hemagglutinin (HA) and sialic acid (SA)—this is an example of a polyvalent interaction. The attachment can be blocked effectively by an inhibitor that is itself polyvalent.



Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors**

Mathai Mammen, Seok-Ki Choi, and George M. Whitesides*

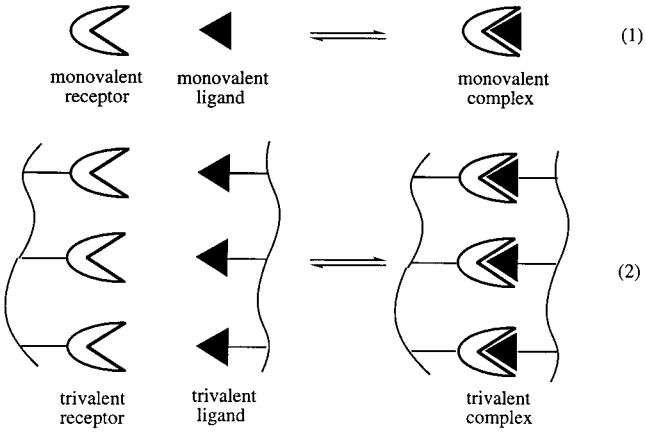
Polyvalent interactions are characterized by the simultaneous binding of multiple ligands on one biological entity (a molecule, a surface) to multiple receptors on another. These interactions occur throughout biology, and have a number of characteristics that monovalent interactions do not. In particular, polyvalent interactions can be collectively much stronger than corresponding monovalent interactions, and they can provide the basis for mechanisms of both agonizing and antagonizing biological interactions that are fundamentally different from those available in monovalent systems. The first part of this review examines the theoretical framework for polyvalency, describes a consistent nomenclature for polyvalent interactions, and discusses the central role of entropy in polyvalency. This examination is qualitative, and selectively highlights the most important characteristics of polyvalency. The second part surveys a number of biological processes that involve polyvalent interactions, with detailed discussion of examples that help to illustrate the many settings for polyvalent systems. This systematic survey suggests new targets and new strategies for the design of pharmaceutical agents based on polyvalency. The third part discusses principles of design, synthesis, and assay of synthetic polyvalent ligands, using the attachment of the influenza virus to an erythrocyte as a prototypical polyvalent system. This discussion of the activities of polyvalent, polymeric inhibitors of the hemagglutination induced by this virus indicates that there are multiple modes of interaction in this representative and particularly well studied polyvalent interaction; the two most important are entropically enhanced binding and steric stabilization.

Keywords: cooperative effects · molecular recognition · noncovalent interactions · polyvalency · receptors

1. Polyvalent Interactions Occur Throughout Biology

The valency of a particle—a small molecule, oligosaccharide, protein, nucleic acid, lipid or aggregate of these molecules; a membrane or organelle; a virus, bacterium, or cell—is the number of separate connections of the same kind that it can form with other particles through ligand–receptor interactions [Eqs. (1), (2)]. This review examines systems in which two biological entities interact oligovalently or polyvalently, that is, through simultaneous, specific association of two or more ligands and receptors.

The idea that many biological systems interact through multiple simultaneous molecular contacts is familiar; it has, however, become a new focus of inquiry in molecular biochemistry as the importance of interactions involving multiple proteins and ligands has begun to be unraveled. The possibility that multiple simultaneous interactions have unique collective properties that are qualitatively different from properties displayed by their constituents, which interact monovalently, suggests new strategies for the design of drugs and research reagents for biochemistry and biology. Enhancing or blocking collective or polyvalent interactions may benefit from (or require) strategies fundamentally different from those used in monovalent molecular interactions.



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[**] A list of the abbreviations used is provided in the Appendix.

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Few biological interactions have so far been established unambiguously to be polyvalent. Thus, here we hypothesize that interactions between biological entities that present multiple ligands and receptors commonly involve polyvalency. This review both summarizes and projects: Its objective is to hypothesize that polyvalency is an important type of interaction in biological systems, and to organize and review experimental information that supports or is relevant to this hypothesis. It also outlines some of the thermodynamic principles that distinguish polyvalent from monovalent interactions, and suggests strategies that are designed specifically to interfere with or promote interactions that are polyvalent.

In Section 3 we discuss the thermodynamics of polyvalent interactions as well as the concepts of cooperativity (using the familiar cooperativity parameter α) and polyvalent enhancement in binding (using β, a new parameter that we believe will be more useful than α for the evaluation of polyvalent inhibitors). In the subsequent sections, we detail interactions in biology that have been well studied and that are certainly or plausibly polyvalent; these interactions serve to illustrate some of the distinct and interesting properties of polyvalent systems. Finally, we discuss the principles and practice of the design of polyvalent ligands, and describe methods of assaying and quantifying the effectiveness of these ligands.

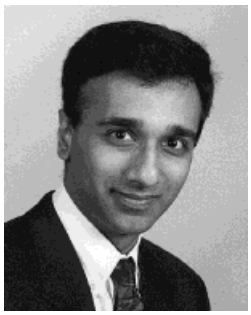
2. Examples of Polyvalent Interactions Relevant to Human Biology

The following examples of polyvalency are drawn from interactions relevant to human biology. The order of categories reflects both the present depth of understanding of these types of interactions and the intensities of the research activities focused on them.

2.1. Adhesion of a Virus to the Surface of a Cell: Influenza and Bronchial Epithelial Cells

In the first step of infection, the influenza virus attaches to the surface of a bronchial epithelial cell (Figure 1).[1, 2] The attachment occurs by interaction between multiple trimers of the hemagglutinin (HA, a lectin that is densely packed on the surface of the virus, about 2–4 per 100 nm^2 or 600–1200 per virus particle) and multiple moieties of *N*-acetylneuraminic acid (=sialic acid (SA), the terminal sugar on many glycoproteins and one that is also arranged densely on the surface of the target cell, about 50–200 per 100 nm^2). Table 1 provides other examples of cell–virus interactions. A growing

G. M. Whitesides



M. Mammen

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George M. Whitesides was born in 1939 in Louiseville, KY. He received his A.B. degree from Harvard College in 1960, and his Ph.D. from the California Institute of Technology with J. D. Roberts in 1964. He was a member of the faculty of the Massachusetts Institute of Technology from 1963 to 1982. He joined the Department of Chemistry at Harvard University in 1982, and was Department Chairman from 1986 to 1989. He is now Mallinckrodt Professor of Chemistry at Harvard University. He has served on many government and industrial councils and advisory boards, is a member of the editorial board for 17 scientific journals, and has received numerous awards including the Arthur C. Cope Award from the American Chemical Society. He is the author of over 600 publications, and his present research interests include biochemistry, surface chemistry, materials science, molecular virology, optics, self-assembly, and organic synthesis.

Mathai Mammen was born in Vellore, India, and raised in Halifax, Nova Scotia. He obtained his B.Sc. Honours in Chemistry from Dalhousie University, and his Ph.D. with G. M. Whitesides from Harvard in 1996. Working in both theory and experiment, he examined aspects of the influence of both entropy and electrostatics on the interaction of systems of molecules. He received his M.D. from Harvard, and was a member of the Harvard Medical School–Massachusetts Institute of Technology Division of Health Sciences and Technology. His research interests include the application of synthetic and biophysical chemistry to the solution of biologically and medically relevant problems (new tools for research, new pharmaceutical constructs, and new medical devices). He co-founded Advanced Medicine, Inc. in South San Francisco, CA and joined in July 1998.

Seok-Ki Choi was born in Puan, South Korea. He received his B.S. degree in Chemistry in 1986 from Seoul National University, where he then obtained his M.S. degree in Organic Chemistry in 1988 with S.-H. Chang. In July 1994, he received his Ph.D. degree in Organic Chemistry under K. Nakanishi at Columbia University in New York. His doctorate research examined the molecular mechanism of action of wasp neurotoxins. Following postdoctoral studies with G. M. Whitesides at Harvard, he joined Advanced Medicine, Inc. in South San Francisco, CA in April 1997. His areas of interest are biological and physical–organic chemistry of natural products and biologically relevant macromolecules.

Table 1. Examples of ligands and receptors[a] on the surfaces of virus and host cells.[3–5]

DNA viruses	Disease/application	Molecule on virus	Molecule on host
Papovaviridae *Polyomavirus* Polyomavirus	DNA-containing tumor virus	VP1[6]	**S**: NeuAc(α2,3)Gal(β1,3)GalNAc[6, 7] **S**: sialyloligosaccharides
Adenovirus *Mastadenovirus* Human adenovirus	acute respiratory disease, pneumonia, gastroenteritis (also a useful vector for gene therapy)	**P**: residues 1–141 of E3gp (fiber knob protein)[8] **P**: RGD base of the penton base protein (different from fiber protein)	**P**: class I MHC[9] **P**: penton base interacts with β_2 integrins[10]
Herpesviridae-alpha *Herpes simplex virus* type 1	herpes simplex	**P**: glycoprotein E and D[11–20]	**P**: gD, gH (proposed two-step attachment)
Varicella–Zoster virus	chicken pox, shingles	**P**: glycoprotein I (gE)[21, 22] (gE dimer resembles Fc receptor)	
Herpesviridae-beta *Cytomegalovirus* Human cytomegalovirus	major cause of infant blindness and retardation; implicated in AIDS and cancer (Kaposi sarcoma)	**P**: gC-11, gp residues 204–297 (86 kDa) of the gene of the H301 α-domain resembling class I MHC α3[23, 24]	**S**: heparan sulfate **P**: class I HLA MHC through β_2-microglobulin[25]
Lymphocryptovirus Epstein–Barr virus	mononucleosis	**P**: gp350[26] **P**: gp3[26]	**P**: C3d receptor CR2 (CD21) of B-lymphocytes; resembling C3 complement fragment C3d[27]
Poxviridae *Orthopoxvirus* Vaccina virus	used for small pox vaccination; similar antigenic patterns to variola virus	**P**: residues 71–80 of the VGF protein; resembling EGF α	**P**: receptor of the epidermal growth factor[27] (there is some evidence to the contrary, however[28]).
Parvoviridae *Parvovirus* Canine Parvovirus	this virus is a model for the human strain, which causes erythema infectiosum ("fifth disease"); the human form may cause hydrops fetalis or fetal death if contracted during pregnancy	**P**: GP1-anchored protein[29]	**P**: 3201 T-cells **P**: surface of precursors of erythrocytes in the bone marrow (infection can cause anemia)
Hepadnavirus Human hepatitis B virus *Hepatitis type B*	serum hepatitis, a chronic infection that results in liver failure and death in 1–2% of cases	**P**: pre-S portion of the Env protein **P**: residues 21–47 of the preS portion of the Env protein **P**: pre-S1 sequence of large S protein[30, 31];	**P**: IL-6 receptor, pre-S1 binding protein **P**: hepatocyte receptor for polymerized serum albumin **P**: hepatocyte receptor for polymeric IgA **P**: sialoglycoprotein **P**: mediated attachment through soluble IL6, gaining entry into nonliver cells indirectly **S**: asialoglycoprotein receptor[32]
Avian hepatitis B virus	serum hepatitis in birds		**P**: glycoprotein 180[33]
RNA viruses	**Disease**	**Molecule on virus**	**Molecule on host**
Picornaviridae *Enterovirus* Poliovirus	polio	**P**: residues 95–105 of the VP1 capsid protein[34]	**P**: member of the immunoglobulin superfamily,[35] not CD44 as previously reported.[36]
Rhinovirus Human rhinovirus	common cold	**P**: residues of VP1 and VP3 major capsid proteins lining a canyon on the virus surface[37–39]	**P**: ICAM-1[40]
Cardiovirus Encephalomyocarditis virus	mengo fever, rarely fatal; pathogenic for many animals (has caused outbreak of fatal myocarditis in pigs, for instance)	**P**: residues of VP1 and VP3 major capsid[41]	**P**: sialoglycoprotein **P**: VCAM-1[42]
Aphthovirus Foot and mouth disease virus	highly infectious disease of cattle, sheep, pigs, and goats (10–70% mortality); usually nonfatal in humans, but readily contracted from animals (causes high fevers)	**P**: NS28 glycoprotein **P**: residues 145–147 (RGD) and 203–213 of VP1 protein **P**: residues 133–158 and C-terminal region of the VP1 protein[37, 38, 43]	**P**: integrins
Reoviridae *Rotavirus* Human rotavirus	single most important cause of gastroenteritis and diarrhea in human infants worldwide: more than 500 million cases annually, resulting in 5 million deaths	**P**: C-terminal portion of hemagglutinin[44–46]	1. **P**: K562 erythroleukemia cells? 2. **P**: β-adrenergic receptor? 3. **P**: sialoglycoproteins in the vili of cells in the small intestine 4. **S**: sialic acids[47, 48]
Togaviridae *Alphavirus* Semliki forest virus	fever, encephalitis	**P**: nucleocapsid	**P**: class I HLA and H-2 MHC molecules[49–51]
Lactate dehydrogenase-elevating virus	fever, encephalitis	**P**: envelope glycoprotein (VP-3P)[52]	**P**: class II 1a MHC molecule of macrophage[53]

Table 1. (cont.)

RNA viruses	Disease/application	Molecule on virus	Molecule on host
Sindbis	related to the western equine encephalitis virus, ca. 5% mortality	**P**: E1-E2[54]	**P**: high-affinity laminin receptor[55, 56]
Rubivirus Rubella	rubella (German measles)		**S**: unidentified glycolipid[57, 58]
Orthomyxoviridae *Influenzavirus* Influenza virus	major cause of various forms of human respiratory disease, including fatal pneumonia	**P**: hemagglutinin protein (HN or H protein)[59–61]	**P**: HN protein, liver cell **S**: sialyloligosaccharides influenza A:[62] **S**: NeuAc(α2,6)Gal(β1,4)GlcNAc, **S**: NeuAc(α2,3)Gal(β1,3)GalNAc, **S**: NeuAc(α2,3)Gal(β1,4)GlcNAc influenza C:[63] **S**: 9-*O*-AcNeuAc
Paramyxoviridae *Paramyxovirus* Sendai virus	as a genus, *Paramyxovirus* is second only to respiratory syncitial virus in causing lower respiratory tract infections in young children	**P**: hemagglutinin protein[64–66]	**S**: sialyloligosaccharides **S**: NeuAc(α2,3)Gal(β1,3)GalNAc **S**: NeuAc(α2,8)NeuAc(α2,3)Gal-(β1,3)GalNAc, **S**: ganglioside GD1a
Newcastle disease virus	lower respiratory tract infections	**P**: hemagglutinin neuraminidase[67]	**S**: sialyloligosaccharides **S**: NeuAc(α2,3)Gal(β1,3)GalNAc[68]
Morbillivirus Measles virus	measles	**P**: measles virus hemagglutinin[69]	**P**: CD46[70]
Pneumovirus Respiratory syncytial virus	leading cause of lower respiratory tract infections (bronchiolitis and pneumonia), possible role in sudden infant death syndrome, major cause of middle-ear infections	**P**: fusion protein; nucleocapsid[71]	
Coronavirus, OC43	common cold, pharyngitis		**S**: $Neu5,9Ac_2$[72]
Rhabdoviridae *Vesiculovirus* Vesicular stomatitis virus		**P**: glycoprotein[73]	**S**: phosphatidylserine **S**: Phosphatidyl inositol **S**: GM_3 ganglioside[74]
Lyssavirus Rabies virus	rabies	**P**: residues 151–238 of G protein, resembling Curare-mimetic neurotoxins[75, 76]	**P**: BHK-21 cells **P**: acetylcholine receptor (residues 173–204 of the α subunit) **S**: sialylated gangliosides
Retroviridae[4, 77] *Oncovirus C—human* Human T-cell leukemia virus (HTLV-1)	cancer—leukemia	**P**: residues 246–253 of the envelope glycoprotein, resembling both the extracellular portion of HLA-B MHC molecule and IL2[78]	**P**: class I HLA MHC molecule **P**: Interleukin-2 receptor
Radiation leukemia virus	cancer—leukemia		**P**: T-cell receptor:L3T4 molecule complex[79]
Amphotrophic retrovirus	cancer—leukemia		**P**: CD34 on marrow progenitor cells[80]
Oncovirus C—Avian Avian leukosis virus	cancer		**P**: low-density lipoprotein receptor[81, 82]
Oncovirus C—Mammalian Murine leukemia virus (Moloney)	cancer—leukemia	**P**: envelope gp71[83]	**P**: lymphoma cell surface IgM **P**: hydrophobic protein (622 amino acids) of unknown function
Ecotropic murine retrovirus	cancer	**P**: envelope glycoprotein[84]	**P**: basic amino acid transporter[85]
Nonecotropic murine leukemia virus	leukemia	**P**: gp70 (surface)[86]	
HIV-1	AIDS	**P**: gp120-resembling Ig heavy-chain regions	**P**: CD4 of T-cell
		P: gp120-resembling neuroleukin	**P**: CD4 molecule interacting with class II HLA-DR MHC molecule
		P: gp120-resembling vasoactive intestinal peptide[87, 88]	**S**: galactosyl ceramide (or closely related molecule on human colon epithelial HT29 cells) **P**: CR2, especially in EBV infected cells **P**: chemokine receptors CXCR-4 (T-cell trophic) and CCR-5 (macrophage trophic; both are seven-transmembrane G-protein coupled receptors[89])
HIV-2	AIDS		**P**: CD4[90]
Simian immunodeficiency virus	immunodeficiency syndrome analogous to AIDS in simians	**P**: SIV mac239[91]	**P**: CD4
African swine virus		**P**: p12, p72[92, 93]	

[a] **P** = protein; **S** = sugar (glycoprotein or glycolipid).

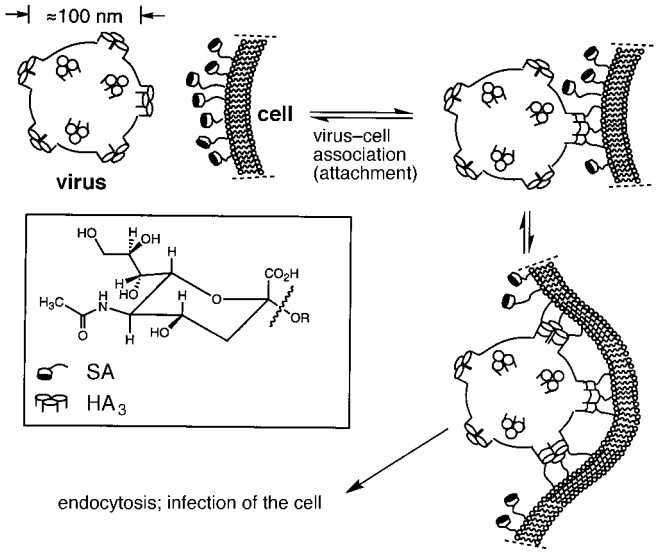


Figure 1. The influenza virus attaches to cells by interaction of trimeric hemagglutinin (HA_3, shown as protruding cylinders on the virus) with sialic acid (SA, shown as caps on the cell). Only a few of the hemagglutinin trimers and SA groups are represented; neither is to scale.

number of viruses are found to use more than one type of interaction for attachment to the target cell.

2.2. Adhesion of a Bacterium to the Surface of a Cell: *E. coli* and Urethral Endothelial Cells

Uropathogenic strains of the bacterium *E. coli* attach both directly and indirectly to the surface of epithelial cells in the urethra and bladder through polyvalent interactions (Figure 2). Several bacterial proteins have been identified that confer this tissue preference. Two examples are P-fimbrae (containing protein G) and type I fimbrae (containing the FimH adhesin), and both are located on the surface of *E. coli*.[94]

We illustrate polyvalency in this system using P-fimbrae as an example. First, these uropathogenic bacteria can use the lectin-like protein G located on the tips of their P-fimbrial filaments to adhere strongly and specifically to multiple copies of the Gal(α1,4)Gal (P_K antigen)[95] portion of a glycolipid present on the surface of the epithelial cells in the urinary tract, especially the kidney. Second, multiple copies of F-protein on the surface of the *E. coli* attach polyvalently to fibronectin, a soluble glycoprotein. The fibronectin, in turn, binds polyvalently to the surface of the epithelial cell. The *E. coli* collect in these tissues of the urinary tract, multiply there, and may cause disease (especially pyelonephritis). In general, bacteria bind either directly to a cellular surface, or to molecules in the extracellular matrix of preferred tissues. Bacteria bind both to sugars and proteins; examples are listed in Tables 2–4.

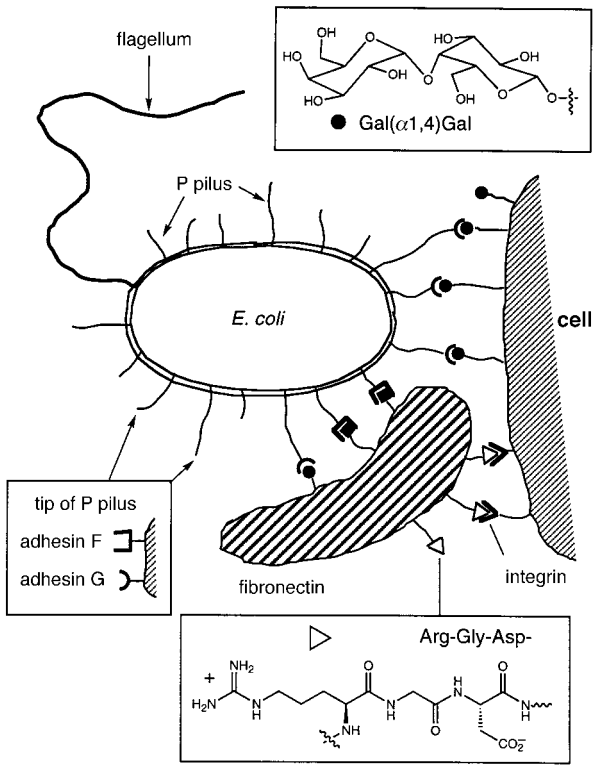


Figure 2. Attachment of uropathogenic *E. coli* bacteria to endothelial urethral cells. This attachment is mediated either by type I fimbrae or by P-fimbrae on the surface of the bacterium. In this example, the bacteria use lectin-like adhesin G located on the tips of their fimbrial filaments (P-fimbrae) to adhere specifically to multiple copies of Gal(α1,4)Gal (P_K antigen) expressed on the surface of the epithelial cells. In addition, multiple copies of adhesin F on the surface of the *E. coli* attach to fibronectin; fibronectin presents multiple copies of Arg-Gly-Asp (RGD) residue to bind tightly to epithelial cell by use of multivalent interactions of RGD and an integrin class of receptor. The illustration (especially, adhesins G and F) is not to scale.

2.3. Binding of Cells to Other Cells: Neutrophil and Arterial Endothelial Cells

The attachment of a neutrophil initially suspended in rapidly flowing blood to the endothelial cells closest to a site of inflammation occurs through polyvalent interactions.[188–190] Signaled by nearby inflammation, this initially rapidly transported neutrophil adheres to the surface of the endothelial cells, and then rolls slowly (10–20 μm min^{-1}); endothelial cells are those that line the interior of the blood vessels (Figure 3). This rolling is mediated by interactions between multiple E- and P-selectins on the surface of the endothelial cell (these selectins are not normally present on the surface of these cells, and are induced by cytokines during inflammation) and multiple glycoproteins displaying sialyl Lewis^X (sLe^X, a tetrasaccharide) on the surface of the neutrophil.[190] In addition, L-selectin, present on the surface of the neutrophil, interacts with sialyl Lewis^X, present on the endothelial cell. The valency of this set of interactions may significantly influence the kinetics, dynamics, and specificity of neutrophil recruitment.[191] Table 5 (page 2763) provides other examples of cell–cell interactions.

2.4. Binding of Cells to Polyvalent Molecules: Bacteria, Antibodies, and Macrophages

All classes of antibodies—one of the key groups of proteins making up the immune system—have multiple equivalent

Table 2. Examples of bacteria that bind sugars (**S**) and proteins (**P**) on the surfaces of host cells.

Bacterium	Disease	Molecule on bacterium	Molecule on host
Actinomyces naeslundii 12104 and A. viscosus LY7	chronic oral disease		**S**: GalNAcβ-containing glycosphingolipids (GSLs), e.g. GalNAc(β1,3)Gal(α)-*O*-ethyl[96, 97]
Candida albicans	part of normal flora of the oral and (female) genital tract, causes disease secondary to a predisposing condition (e.g. diabetes mellitus, immunodeficiency, long-dwelling catheters, antimicrobials that eliminate normal bacterial flora)	**P**: laminin receptor (37 kDa) **P**: fibrinogen-binding mannoprotein (58 kDa) **P**: C3d receptor **P**: Ubiquitin[98]	**S**: fucose-containing glycosides[99] on epithelial surface
Chlamydia trachomatis	chronic keratoconjunctivitis that can proceed to scarring and blindness, sexually transmitted diseases, respiratory infections	**S**: polysaccharide containing 7–9 mannose residues[100]	**P**: mannose-binding proteins[100]
Enteroaggregative *E. coli* (*EAggEC*)	food poisoning	**P**: hemagglutinins[101]	**S**: sialic acid
Uropathogenic *E. coli*	urinary tract infection	**P**: PapG adhesin[102] **P**: FimH adhesin[103]	**S**: Gal(α1,4)Gal on glycolipid **P?**: uroplakins Ia and Ib
S-fimbriated *E. coli*	cerebral vascular complications of meningitis		**S**: NeuGc(α2,3)Gal and NeuAc(α2,8)NeuAc[104] **S**: NeuGc(α2,3)Gal and NeuAc(α2,8)NeuAc[105] (found on endothelial cells of brain microvasculature)
Helicobacter pylori	gastroduodenal ulcers, gastric cancers	**P**: hemagglutinin **P**: laminin-binding protein	**S**: Lewis(b) blood group antigen[106] **S**: Neu5Ac(α2,3)Gal (SA-dependent strains)[107, 108]
Mycoplasma pneumoniae	Pneumonia	**P**: protein (40 and 90 kDa) on the tips of the pili[109]	**S**: long-chain sialo-oligosaccharides[110, 111]
Mycoplasma bovis	pneumonia, genital and urinary tract infection	**P**: protein (26 kDa)[112]	**S**: sialic acid residues and probably also sulfatide groups
Neisseria menigitidis Meningococcus	meningitis, acute adrenal insufficiency	**P**: pili (unusual in that pili contain digalactosyl-2,4-diacetamido-2,4,6-trideoxyhexose)[113]	**P**: CD66 on epithelial cells and neutrophils[114] **P**: glycoprotein G of RSV on the surface of the target cell (coinfection)[115]
Porphyromonas (Bacteroides) gingivalis	gingivitis		**S**: D-GalNAc[116]
Pseudomonas aeruginosa	colonizes regions devoid of natural defenses (catheters), and causes disease in immunodeficient hosts	**P**: pilus adhesin	**S**: GalNAc(β1,4) in asialo-GM1 and asialo-GM2 or GM1[117] **S**: salivary mucin glycopeptides (sialic acid)[118] **S**: lactose of glycolipids[119]
Rhizobium lupini		**P**: L-fucose-binding protein	**S**: L-fucose[120]
Staphylococcus saprophyticus	urinary tract infection	**P**: surface lectins	**S**: blood group A (terminal GalNAc)[121]
Streptococcus suis	meningitis		**S**: Gal(α1,4)Gal present in the P1 and Pk blood group antigen[122] **S**: N-Ac(α2 $\rightarrow$3poly-NAc-lactosamine glycans; NeuNAc(α2,3)Gal(β1,4)GlcNAc[123]
Streptococci (M+ group A)	nasopharyngitis; release of erythrogenic toxins can cause scarlet fever; can occur as secondary infection to influenza virus, and ultimate cause of fatal pneumonias in these cases		**P**: C3, mainly C3b and iC3b on PMN[124]
Streptococcus sanguis, *Streptococcus sobrinus* (oral cavity)	part of normal flora in the upper respiratory tract: *deficiency* can cause disease		**S**: sialic acid of salivary glycoproteins[125]
Yersinia enterocolitica	severe abdominal pain and diarrhea; can cause fatal sepsis in some cases		**S**: Gal, GalNAc, Lac in intestinal mucin[126]

receptor sites: two (IgD, IgE, IgG, IgA), four (IgA), six (IgA), or ten (IgM). Polyvalent binding to the structures that these antibodies recognize—antigens or other ligands present on the surfaces of bacteria, viruses, parasites, drugs, "nonself" cells, or other structures including noncovalent complexes not usually present in the blood circulation—seems to be an ubiquitous characteristic of immune recognition. These interactions may both inhibit processes important to infection (e.g., attachment of a foreign organism to target cells) and promote clearance (removal of the foreign particles either by

Table 3. Examples of bacteria that bind to the derivatives of the glycolipid lactosylceramide.[119, 122, 127–133]

Bacterium	Target tissue
Bacteroides fragilis	large intestine
Bacteroides ovatus	large intestine
Bacteroides vulgatus	large intestine
Bacteroides distasonis	large intestine
Bacteroides thetaiotamicron	large intestine
Lactobacillus fermentum	large intestine
Lactobacillus acidophilus	various places
Fusobacterium necrioohorus	large intestine
Fusobacterium varium	large intestine
Clostridium difficile	large intestine
Clostridium botulinum	large intestine
Propionibacterium granulosum	skin, large intestine
Propionibacterium acne	skin
Propionibacterium freudenreichii	milk products
Actinomyces viscosus	mouth
Actinomyces naeslundii	mouth
Shigella dysenteriae	large intestine
Shigella flexnerii	large intestine
Shigella sonnei	large intestine
Salmonella typhimurium	large intestine
E. coli	intestine
Vibrio cholerae	small intestine, large intestine
Campylobacter jejunii	intestine
Hemophilus influenzae	respiratory tract
Yersinia pseudotuberculosis	intestine
Yersinia pestis	intestine
Neisseria gonorrhoeae	genital tract
Pseudomonas aeruginosa	respiratory tract

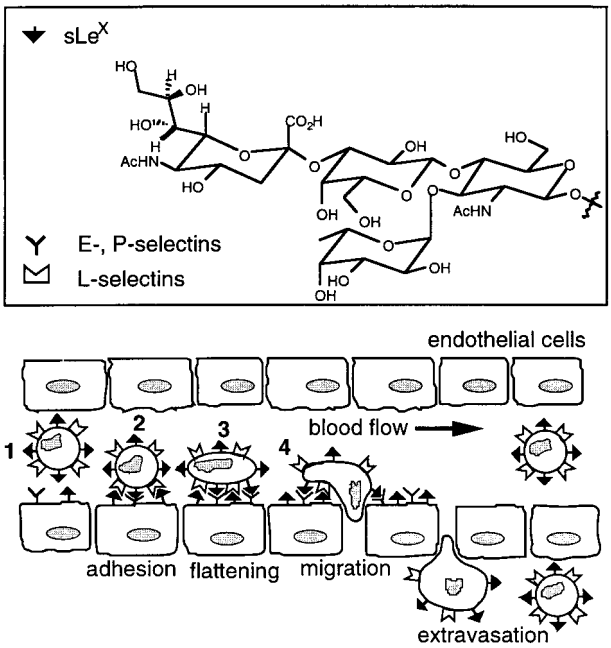


Figure 3. Injury results in expression and display of E- and P-selectins on the surface of nearby endothelial cells. Neutrophils (**1**) are attracted to this site (**2**). The neutrophil interacts polyvalently (through sLe^X moieties) with E- and P-selectins. In addition, L-selectins, expressed on the surface of the neutrophil, interact with sLe^X moieties present on the endothelial cell. Neutrophils change their shape upon attachment (**3**). Further adhesion mediated by integrins leads to extravasation (**4**).

Table 4. Examples of bacteria that bind sugars (**S**) and proteins (**P**) in the extracellular matrix.

Bacterium	Disease	Ligand (receptor) on the bacterium	Ligand (receptor) on the host matrix
Aspergillus fumigatus conidia	various localized fungal infections		**P**: D domains of the fibrinogen molecule[134]
Borrelia burgdorferi	lyme disease		**P**: integrin αIIbβ3 (glycoprotein IIb-IIIa) (RGD)[135]
Bordetella pertussis	whooping cough (pertussis)	**P**: pertactin and filamentous hemagglutinin (FHA); cell-binding sequence (RGD)[136]	
Candida albicans	part of normal flora of the oral and (female) genital tract, causes disease secondary to a predisposing condition (e.g. diabetes mellitus, immunodeficiency, long-dwelling catheters, antimicrobials that eliminate normal bacterial flora)	**P**: 37-kDa laminin receptor **P**: fibrinogen-binding mannoprotein (58 kDa) **P**: C3d receptor **P**: ubiquitin[98]	**S**: cell-wall polysaccharide of *Streptococcus gordonii* during coinfection[137] **S**: various oligosaccharides containing both β-1,2 and α-1,2 linkages[138–140]
Chlamydia trachomatis	chronic keratoconjunctivitis that can proceed to scarring and blindness, sexually transmitted diseases, respiratory infections	**P**: heparin-binding protein[141]	**S**: heparan sulfate-like GAG[141, 142] with a decasaccharide of minimum chain length[143]
Enterococcus faecalis	diarrhea		**S**: galactose, fucose, and mannosamine, but not mannose[144]
E. coli with S fimbriae	gastrointestinal infections	**P**: S-fimbriae protein SfaA	**S**: inhibited by NeuAc(α2,3)lactose[145] **S**: glycolipids containing terminal Gal($3SO_4$)β-1 residues[132]
Haemophilus influenzae	upper respiratory infections, often secondary to influenza virus	**P**: proteins of high molecular weight (HMW-1 and HMW-2)[146]	
Leishmania donovani	protozoan parasite causing kala-azar (visceral leishmaniasis)	**P**: heparin-binding protein	**S**: heparin[147]
Mycobacterium paratuberculosis	not a major human pathogen		**P**: fibronectin[148, 149]
Mycobacterium bovis	chronic granulomatous infections, especially in the lung	**P**: protein 85B and p55 protein	**P**: collagen-binding domain of fibronectin, glycoprotein present in plasma[150]

Table 4. (cont.)

Bacterium	Disease	Ligand (receptor) on the bacterium	Ligand (receptor) on the host matrix
Mycobacterium avium-intracellulare (MAI)	a common pathogen in AIDS patients		**P**: laminin, collagen I, and fibronectin[151]
Prevotella intermedia	periodontal infection	**P**: lactoferrin-binding protein (lactoferrin inhibits the binding)	**P**: fibronectin, collagen of type I and type IV, and laminin[152]
Salmonella enteritidis	severe diarrhea (common food poison from chicken and chicken eggs)		**P**: fibronectin[153]
Staphylococcus aureus	major human pathogen responsible for wide range of infections, can proceed to meningitis, endocarditis, and osteomyelitis	**P**: three repeats of a 38-residue D motif **P**: fibronectin-binding proteins (galel A and ZZ-FnBP B) **P**: protein (60 kDa) **P**: collagen-binding domain (CBD) of collagen adhesin **P**: collagen receptor containing either two or three copies of a repeat motif with 187 amino acids	**P**: N-terminal fragment (29 kDa) of fibronectin (Fn29 K)[154] **P**: fibronectin[155] **P**: bone sialoprotein (BSP) (small, ca. 80 kDa) integrin-binding, RGD-containing bone matrix glycoprotein[156, 157] **P**: collagen of cartilage[158] **P**: collagen[159, 160] **P**: N-terminal region (heparin-binding domain) of fibronectin[161]
Staphylococcus aureus	osteomyelitis and infectious arthritis	**P**: collagen adhesin **P**: fibrinogen receptor (clumping factor) fibronectin-binding protein (FnBP) **P**: unknown protein (60 kDa)	**P**: collagen (high degree of specificity and affinity)[162] **P**: fibrinogen[163, 164] **P**: laminin (the pepsin-derived (P1) fragment)[165, 166] **P**: vitronectin[167]
Staphylococcus saprophyticus	urinary tract infections		**S**: adherence is partially inhibited by mannose[168]
Streptococcus	wide range of gastrointestinal and respiratory infections		**P**: collagen type I[160]
Group A streptococci	infective endocarditis, glomerulonephritis, and rheumatic fever	**P**: lipoteichoic acid (LTA) and M protein (binding is inhibited by LTA)[169, 170] **P**: glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[171, 172] **P**: protein F[173] **P**: PAM (related to the M proteins) **P**: M3 protein[174]	**P**: fibronectin **P**: human plasminogen and plasmin **P**: multiple binding to plasma fibrinogen, albumin and fibronectin
Nephritis(+) and nephritis(-) group A streptococci	glomerulonephritis	**P**: nephritis plasmin binding protein (NPBP)[175]	**P**: human plasmin (the binding is blocked by ε-aminocaproic acid)
Streptococcus bovis	urinary tract infections, endocarditis	**S**: lipoteichoic acid derivative[176]	
Streptococcus defectivus	not a human pathogen	**P**: surface protein (ca. 200 kDa)	**S**: cell-secreted extracellular matrix (ECM)[177]
Streptococcus dysgalactiae	neonatal sepsis and meningitis	**P**: fibronectin (Fn) receptors FnBA and FnBB	**P**: fibronectin[156, 157]
Streptococcus pneumoniae	pneumonia		**P**: laminin; collagen types I, II, and IV; fibronectin; and vitronectin[178]
Streptococcus pyogenes	major pathogen responsible for a wide range of systemic and local infections, and associated with post-streptococcal immunological disorders	**P**: protein F (X-ray structure known) **P**: streptococcal fibronectin-binding protein (Sfb protein, sequence of 37 amino acids)[179, 180] **P**: glycosaminoglycan-binding protein (GAG-BP; 9 kDa)[181] **P**: M protein[182, 183]	**P**: fibronectin[148, 184] **P**: basal laminae of human cardiac muscle
Veillonella atypica PK1910 (oral bacterium)	oral infections		**S**: co-aggregations with certain human oral streptococci (both lactose-inhibitable and lactose-non-inhibitable)[185]
Yersinia enterocolitica	abdominal pain and diarrhea	**P**: membrane protein YadA	**P**: cartilage-derived human cellular fibronectin and human plasma fibronectin[186]
Yersinia pseudotuberculosis	abdominal pain and diarrhea	**P**: adhesin YadA	**P**: β1 integrins[187]

degradation by macrophages and other components of the immune system, or by filtration by the kidney).[207] Polyvalency is used here for high-affinity binding to surfaces that have repeated epitopes, a defining characteristic of the surfaces of almost all invading pathogens.

Mannose residues on the tail (the Fc portion) of the antibody interact with mannose receptors (the Fc receptor) on the surface of a macrophage (a type of white blood cell important for clearing infectious particles). The interaction of a *single* Fc portion with its receptor seems to be too weak to

Table 5. Examples of polyvalent cell–cell interactions.

Cell 1	Molecule on cell 1	Cell 2	Molecule on cell 2
neutrophil	L-selectin, P-selectin[192–194]	endothelial cell	sulfated sLe^X[193, 195]
neutrophil	sulfated sLe^X[196]	endothelial cell	E-selectin[196]
neutrophil	E-selectin,[193] cadherins[198]	neutrophil	sulfated sLe^X[193]
neuron	neural-cell-adhesion molecule (NCAM) such as L1, NCAM-H, CD24, and α_6-integrin[192, 197]	endothelial cell	E-selectin[196]
T-cell	T-cell receptor[199]	antigen-presenting cells	MHC[199]
T-cell	CD3[200]	antigen-presenting cells	MHC
T-cell	CD28[201]	antigen-presenting cells	MHC
platelet	Ia/IIa	endothelial cell	collagen[202]
platelet	IIb/IIIa[203]	platelet	IIb/IIIa
tumor cell	GalTase[204]	endothelial cell	GlcNAc
sperm	GalTase[204]	egg	GlcNAc
aging red blood cell	desialylated glycoproteins[205, 206]	hepatocyte	C-type lectin

induce a response by the macrophage; that is, free (uncomplexed) antibody in solution does not activate macrophages, nor does a single antibody bound to a degraded piece of foreign pathogen. However, *multiple* antibodies bound to the surface of an infecting particle do interact strongly with multiple receptors on the surface of the macrophage, and give a three-layered structure stabilized at both interfaces through polyvalent interactions (Figure 4).[208–210]

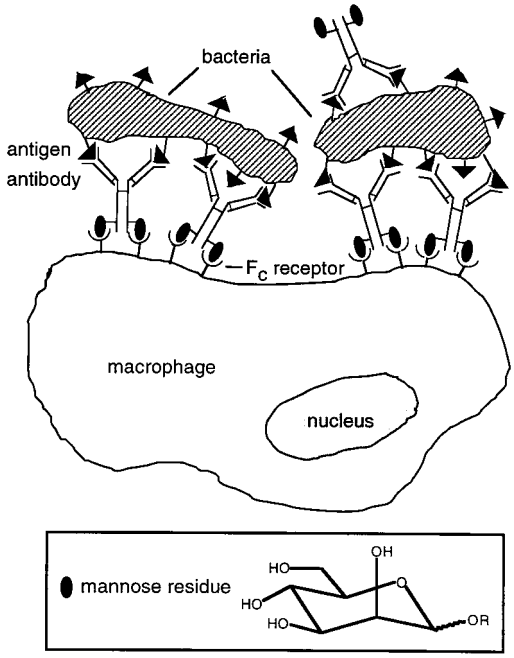


Figure 4. Layered polyvalent binding of a macrophage to bacterium (or other pathogens) through antibodies. The antibody recognizes a surface ligand (usually a protein) on the surface of bacterium, and can bind bivalently. The macrophage recognizes (with Fc receptors) the mannose residues on the constant region "tail" of the antibody, and also binds multivalently to the antibody-decorated pathogen.

Furthermore, not only does polyvalency in this system permit stability and specificity in the recognition of a bacterium by a macrophage, but subsequent action by the macrophage is also critically dependent on polyvalent interactions. Multiple interactions between the macrophage and the antibody-coated bacterium lead to a cross-linking of the surface receptors on the macrophage, triggering an internal signal in the macrophage to ingest (phagocytose) the bacterium which then leads to its degradation.[211] In Section 4.3.1, we discuss a general mechanism for this sort of polyvalent signal transduction. Other examples of polyvalent molecules that attach to the surfaces of cells are given in Tables 6 and 7.

2.5. Binding of Polyvalent Molecules to Polyvalent Molecules: Binding of Transcription Factors to Multiple Sites on DNA

An example of molecules that interact polyvalently is taken from the biology of gene regulation by oligomeric transcription factors (Figure 5). The retinoid X receptor (RXR) functions as a transcription factor in the presence of its ligand.[258] Each RXR–ligand complex (RXR-L) binds a DNA single strand called the cellular retinol-binding protein II element (CRBP-II element). The intrinsic affinity of one unit of RXR-L for one CRBP-II element is low; that of a dimer $(RXR\text{-}L)_2$ for two adjacent elements $(CRBP\text{-}II)_2$ is higher, but still low; that of a tetramer $(RXR\text{-}L)_4$ for four adjacent elements $(CRBP\text{-}II)_4$ is moderate; and that of a pentamer $(RXR\text{-}L)_5$ for five adjacent elements $(CRBP\text{-}II)_5$ is high, and significantly higher than that of the tetramer. In this example, the transcription factor is functioning as a polyvalent aggregate of ligands, and the DNA containing multiple binding sites is a polyvalent receptor (examples are given in Table 8). Polyvalency of this sort creates a transcriptive response that is highly sensitive to the concentration of the transcription factor: The rate of transcription is proportional to $\Sigma_n C_n[\text{CRBP-II}]^n$, where C_n are constants.

Since the rate of transcription is mostly likely highest with the pentamer, the rate is approximately proportional to $[\text{CRBP-II}]^5$; this dependence on concentration is much higher than is possible with monovalent interactions (for which the

Table 6. Receptors for bacterial toxins.[127, 129, 212, 213]

Bacterium	Toxin	Ligand[a]
Vibrio cholerae	cholera toxin[214]	**S**: GM_1: Gal(β1,3)GalNAc(β1,4)(NeuAc(α2,3))Gal(β1,4)Glc(β)-ceramide[215, 216] **S**: isoligands:[216] NeuAc(α2,3)Gal(β1,3)GalNAc(β)(NeuAc(α2,3)Gal(β1,4)Glc(β)-ceramide, Gal(β)GalNAc(β1,4)(NeuAc(α2,8)NeuAc(α2,3)Gal(β1,4)Glc(β)-ceramide, GalNAc(β1,4)-Gal(β1,3)GalNAc(β1,4)((NeuAc(α2,3))Gal(β1,4)Glc(β)-ceramide, Fuc(α1,2)Gal(β1,3)-GalNAc(β1,4)((NeuAc(α2,3))Gal(β1,4)Glc(β)-ceramide, Gal(β1,3)GalNAc(β1,4)-(R-NeuAc(α2,3)Gal(β1,4)Glc(β)-ceramide (R = Lucifer yellow CH, rhodamine, or DNP)
E. coli	heat-labile toxin[217]	**S**: GM1[145]
Clostridium tetani	tetanus toxin	**S**: Gal(β1,3)GalNAc(β1,4)((NeuAc(α2,8))NeuAc(2,3)Gal(β1,4)Glc(β)-ceramide[218] **S**: isoligands: NeuAc(α2,3)Gal(β1,3)GalNAc(β1,4)((NeuAc(α2,8))NeuAc(α2,3)-Gal(β1,4)Glc(β)-ceramide, NeuAc(α2,8)NeuAc(α2,3)Gal(β1,3)GalNAc(β1,4)(NeuAc(α2,8)-NeuAc(α2,3)Gal(β1,4)Glc(β)-ceramide
Clostridium botulinum	botulinum toxin A and E[219]	**S**: NeuAc(α2,8)NeuAc(α2,3)Gal(β1,3)GalNAc(β1,4)(NeuAc(α2,8))NeuAc(α2,3)-Gal(β1,4)Glc(β)-ceramide
Clostridium botulinum	botulinum toxin B, C, and F[219]	**S**: NeuAc(α2,3)Gal(β1,3)GalNAc(β1,4)(NeuAc(α2,8))NeuAc(α2,3)Gal(β1,4)Glc(β)-ceramide
Clostridium botulinum	botulinum toxin B[220]	**S**: Gal(β)-ceramide
Clostridium perfringens	delta toxin[213]	**S**: GalNAc(β1,4)(NeuAc(α2,3))Gal(β1,4)Glc(β)-ceramide
Clostridium difficile	toxin A[221]	**S**: Gal(α1,3)Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc(β)-ceramide
Clostridium difficile	shiga-like toxin (SLT)-I and SLT-II/IIc[222]	**S**: Gal(α1,4)Gal(β) (P1 disaccharide), Gal(α1,4)Gal(β1,4)GlcNAc(β) (P1 trisaccharide), or Gal(α1,4)Gal(β1,4)Glc(β) (Pk trisaccharide)
Shigella dysenteriae or *E. coli*	shiga toxin[213, 222]	**S**: Gal(α1,4)Gal(β)-ceramide
Shigella dysenteriae or *E. coli*	vero toxin[223]	**S**: Gal(α1,4)Gal(β1,4)Glc(β)-ceramide
Bordella pertussis	pertussis toxin[224, 225]	**S**: NeuAc(α2,6)Gal[224, 225]
S. Dysenteriae I	dysenteriae toxin[128]	**S**: GlcNAc(β1)

[a] **S** = sugar.

Table 7. Examples of cross-linking of surface receptors as a mechanism of signal transduction.[a]

Process	Receptor – ligand
degranulation of mast cells (allergy)	allergens oligomerize IgE on the surface of the mast cell (P, N:N)[226–230]
cellular differentiation and growth (developmental biology: differentiation, migration, apoptosis)	fibroblast growth factor (FGF) dimerizes FGF receptor; a role for heparin in forming a polyvalent template for FGF and other heparin-binding growth factors is emerging (P, N:N)[231–235] epidermal growth factor (EGF) dimerizes EGF receptor (NP)[236] cytokine stem cell factor (SCF) dimerizes kit receptor on the surface of the stem cell (P, 1:2)[237] transforming growth factor (TGF) dimerizes TGF receptor (NP)[238] platelet-derived growth factor (PDGF) dimerizes PDGF receptor (P, 1:2)[239] a single human growth hormone (hGH) dimerizes hGH receptor (P, 1:2)[240–242] hepatocyte growth factor (HGF) dimerizes c-Met receptor; heparin may provide a polyvalent template for HGF (see FGF above; P, N:N)[243] EPO dimerizes EPO receptor on erythrocyte precursor cell (P, N:N)[244] vascular endothelial growth factor (VEGF) binds to VEGF Receptor Flt-1 (NP)[245] IL6 hexamerizes IL6 receptor (P)[246] IL4 dimerizes its receptor (P, 1:2)[247] human macrophage colony stimulating factor (M-CSF) dimerizes M-CSF receptor (P, 1:2)[248] lactogenic hormone can dimerize prolactin receptors (PRLR) (P, 1:2)[249] two monovalent granulocyte colony stimulating factors (G-CSF) stimulate the dimerization of two G-CSF receptors (NP)[250]
compaction during early embryogenesis	receptor for X hapten and multivalent X hapten (free multivalent versions of X hapten decompact; monovalent X haptens have no effect; P, N:N)[251]
parasympathetic response (autonomic nervous system)	acetylcholine receptor undergoes heterodimerization by unknown mechanism (NP?)[252]
acrosomal reaction in the interaction of sperm with egg (reproductive biology)	GalTase on sperm, GlcNAc on the exterior of the egg (P, N:N)[253]
peptide-independent clonal expansion of B-cells (immunology)	antibody on the surface of a B-cell; polysaccharide coats on the surface of microorganisms (P, N:N)[254]
interferon actions	interferon-alpha (IFN-alpha) dimerizes its receptor (P, N:N)[255]
T-cell clonal expansion	T-lymphocyte receptors CD28 and CTLA-4 bind and are oligomerized by co-stimulatory molecules CD80 (B7 – 1) and CD86 (B7 – 2) on antigen presenting cells (P, N:N)[256]
bacterial chemotaxis	aspartate dimerizes bacterial receptor Tar (NP)[257]

[a] P, 1:2 = Heterobivalent ligand dimerizes two surface receptors; P, N:N = *N*-valent ligand oligomerizes *N* surface receptors; P = probably polyvalent, but with unknown ligand – receptor stoichiometry; NP = possibly a non-polyvalent mechanism.

Table 8. Examples of transcription factors that bind polyvalently to DNA.[259–261]

Transcription factor	Number of sites that recognize DNA
BZip-type transcription factors	2 (noncovalent, homodimers)[262]
lambda repressor	2 (noncovalent, homodimers)[263]
p53	4 (noncovalent, homotetramers)[264]
RXR to CRBP-II	4–5 (noncovalent, oligomers)[265]
estrogen receptor	2 (noncovalent, homodimers; the dimer is induced by estradiol and binds DNA 50 times more rapidly than the monomer)[266]
thyroid hormone receptor	2 (noncovalent, homodimers or noncovalent heterodimer with 9-*cis*-retinoic acid receptor; the dimer is induced by binding to thyroid hormone)[267]
Zn fingers	1–3 (covalent, heterooligomers)[268, 269]

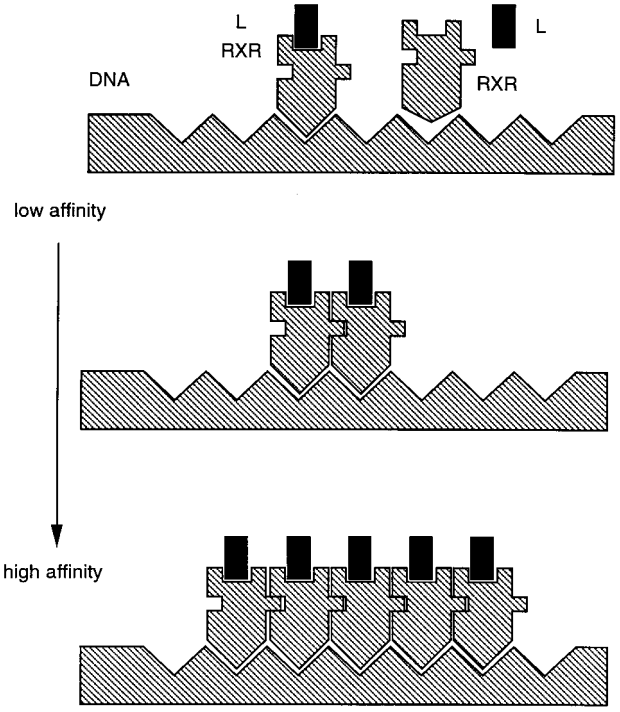


Figure 5. Binding of transcription factors to multiple sites on DNA. Top: The complex of monomeric retinoid X receptor (RXR) and ligand (L), RXR-L, binds with low affinity to the cellular retinol-binding protein II element (CRBP-II) on DNA. Middle: The dimeric complex $(RXR\text{-}L)_2$ has higher affinity than the monomeric complex. Bottom: The pentameric complex $(RXR\text{-}L)_5$ has very high affinity for DNA.

rate of transcription would be proportional to the concentration of the monovalent transcription factor, [CRBP-II]).

3. Nomenclature and Thermodynamics

3.1. Free Energy and Binding Constants in *N*th Order Polyvalent Interactions

We start with nomenclature. In chemistry, it is universal to think of molecular recognition in terms of two components: a protein receptor and a species recognized by the receptor (a ligand, inhibitor, substrate, epitope, or some other molecule or portion of molecule, depending on the context); here we will use the word ligand for this second species. In chemical nomenclature, the receptor is a protein with a declivity or pocket on its surface; the ligand is the molecular entity that fits into that pocket [see Eq. (1) on page 2755]. The receptor is the lock; the ligand is the key. In the literature of cell-surface biology, usage has not been consistent, and both the receptor and the ligand have been called the receptor, with the convention that the receptor was the species on the surface of the target cell. Wherever possible, we will use the chemical system of nomenclature: In this review, the receptor is the protein with a surface declivity that participates in specific recognition.

For polyvalent interactions as a class, there is no accepted nomenclature. Because there are a number of different ways in which N receptor sites can interact with N ligands, and because the free energy of binding of an interaction depends strongly on its details, there is probably no simple general nomenclature. We will use the least complex system of nomenclature that we can. We will call an interaction between N ligands and N receptors distributed on two entities an Nth-order polyvalent interaction that occurs with free energy of association ΔG_N^{poly}. Equation (2) illustrates a third-order polyvalent interaction that occurs with free energy of association ΔG_3^{tri}. The proposed nomenclature for polyvalent interactions is summarized in Figure 6.

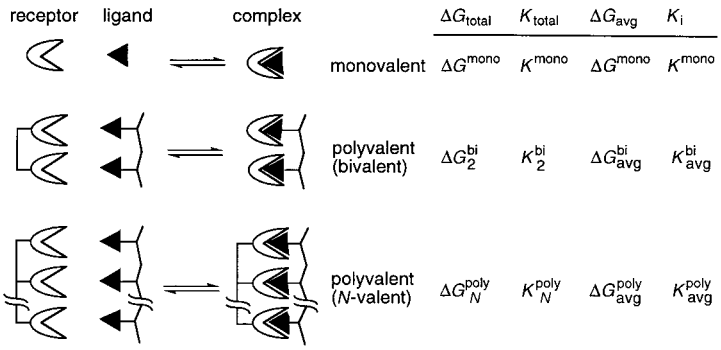


Figure 6. Proposed nomenclature for the polyvalent interactions; relationships between free energies of binding (ΔG) and inhibition constants (K_i) for both monovalent and polyvalent systems.

The average free energy of interaction, ΔG_{avg}^{poly}, between a single ligand moiety and a single receptor moiety in the polyvalent interaction shown in Figure 6 is equal to $\Delta G_N^{poly}/N$ [Eq. (3)]. A monovalent ligand–receptor interaction occurs

$$\Delta G_{avg}^{poly} = \Delta G_N^{poly}/N \quad (3)$$

$$\Delta G = -RT\ln(K) \quad (4)$$

$$K_N^{poly} = (K_{avg}^{poly})^N \quad (5)$$

with free energy change ΔG^{mono}; N monovalent, independent receptors interact with N monovalent, independent ligands

with a free energy change of $N\Delta G^{mono}$. The most useful comparisons of free energies of association involve the same numbers of ligands and receptors, and must be considered thoughtfully if different numbers of ligands and receptors (and different units for equilibrium constants) are involved. Equations (3) – (5) give the values of the corresponding association constants K.

Other terms have been used in the literature to refer (often imprecisely and inconsistently) to the thermodynamics of polyvalent interactions. It may be useful to review these terms briefly, and to incorporate them into the nomenclature we introduce in this manuscript.

"Affinity" is a qualitative term, and "affinity constant" its quantitative associate used correctly to mean association constant K. "Avidity" is a term whose origin is unclear, but refers to the association constant of a polyvalent interaction. We would precisely define avidity as the quantity given by Equation (5), K_N^{poly}. As an example, a Fab fragment (the portion of one arm of an antibody that contains the ligand binding site) binds a ligand with a certain affinity K. The intact antibody is a covalent collection of more than one Fab fragment (IgG, for example, has two Fab fragments). The binding of bivalent IgG to a dense surface of ligand can be imagined conceptually as the binding of one monovalent entity to the same surface, and described by a different association constant. That association constant, K_2^{bi} in this review, is referred to as the avidity of the bivalent antibody. In many instances, the avidity is found to be greater than the affinity K of the component monovalent interactions (Fab fragment). The extent to which K_2^{bi} is greater than K depends sensitively on both the structure and geometry of the antibody and the precise arrangement of the ligands on the surface. Since avidity has traditionally only been used to refer to polyvalent interactions where $N \approx 2-10$, and not for $N \approx 10^5$, we will not use this term in this manuscript because we do not want to conplicate further an already confusing system of nomenclature. We will use the terms defined by Equations (3) – (5) consistently.

The "chelate effect" had its origin in chemistry, and is often used in organometallic chemistry to refer qualitatively to the enhanced binding of electron donor groups (like an amine) for a metal (like iron) when the donor groups are joined covalently by some linking group. Again in this review, the chelate effect refers to those instances when K_N^{poly} is greater than K of any of the component interactions in the analogous fully monovalent binding event. As an example, K would refer to the association constant K for the interaction of methylamine with iron, and K_2^{bi} is the association constant of ethylenediamine with iron. The chelate effect refers to the observation that K_2^{bi} is often greater than K. The degree to which K_2^{bi} is larger than K is highly dependent on the structure and geometry of the linking group.

3.2. Cooperativity: The Magnitude of α

The average free energy of interaction between a ligand moiety and receptor moiety in a polyvalent interaction (ΔG_{avg}^{poly}) can be greater than, equal to, or less than the free energy in the analogous monovalent interaction [ΔG^{mono}; Eqs. (6) – (8)]. Following accepted nomenclature in biochem-

$$\Delta G_{avg}^{poly} = \alpha \Delta G^{mono} \quad (6)$$

$$N\Delta G_{avg}^{poly} = \Delta G_N^{poly} = \alpha N \Delta G^{mono} \quad (7)$$

$$K_N^{poly} = (K_{avg}^{poly})^N = (K^{mono})^{\alpha N} \quad (8a)$$

$$\alpha = \frac{\lg(K_N^{poly})}{\lg(K^{mono})^N} \quad (8b)$$

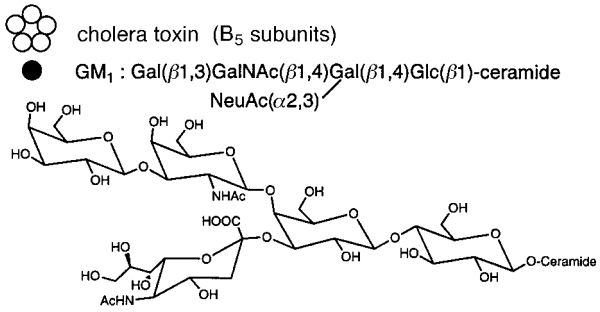
istry, we call these classes of polyvalent interactions positively cooperative (synergistic), noncooperative (additive), or negatively cooperative (interfering), respectively. We follow the convention of defining α as the degree of cooperativity.[270] The units of α depend on the order of the polyvalent interaction.

Cooperativity in biology has been rigorously defined and reviewed previously.[271, 272] The best studied positively cooperative systems in biology do not involve polyvalency. For example, the binding of four O_2 molecules to tetrameric hemoglobin occurs with cooperativity; that is, the free energy of binding of the second oxygen molecule to hemoglobin is more favorable than the binding of the first, and $-\Delta G_{avg}^{poly} > -\Delta G_{(first)}^{mono}$. The degree of cooperativity, α, in such non-polyvalent systems is greater than one and unitless.

There are presently no convincingly characterized examples of positive cooperativity for *polyvalent* systems in the literature. As a class, to our knowledge, polyvalent interactions have not been quantified sufficiently frequently or carefully for positive cooperativity to be inferred unambiguously in even one system.[273]

One example of a positively cooperative polyvalent interaction ($\alpha > 1$) *may* be the association of pentameric cholera toxin with GM_1, an oligosaccharide portion of the GM_1 ganglioside (Figure 7). Cholera toxin consists of five subunits (AB_5). The binding of the toxin to *monomeric* GM_1 (GM_1 that has been cleaved from the ceramidyl moieties on the cellular surface) provides a well-studied example of positive cooperativity through purely enthalpically enhanced binding. As will be discussed shortly, the entropy of the first binding event of a monomeric GM_1 derivative to pentameric cholera toxin is equal to the entropy of each subsequent binding event. All differences in the free energies of binding (Figure 7) of monomeric GM_1 to the toxin are therefore due to differences in enthalpy. Schoen et al. performed calorimetric studies of the binding of pentameric B_5 to five independent GM_1 oligosaccharide units in solution.[274] They found that the binding constant of the first ligand was *lower* than the binding constant of the second by a factor of 4. Calculated purely statistically, the binding constant of the first ligand would be expected to be *greater* by a factor of 5/2 than the second binding constant.[275] We conclude that the binding was enhanced enthalpically.

Schoen et al. also report that the binding of pentavalent B_5 to the surface of a cell that is densely covered with GM_1 moieties occurs essentially irreversibly and with greater ΔG of association than the binding of pentameric cholera toxin to five monomeric units of GM_1. Since the enthalpy of interaction is approximately the same for single (monovalent) GM_1 and for GM_1 immobilized on a surface, this difference in



State	No. of ligands (l)	Configuration	$l\Delta G - j\Delta q$
1	0		0
2	1		ΔG
3	2		$2\,\Delta G$
4	2		$2\,\Delta G - \Delta q$
5	3		$3\,\Delta G - \Delta q$
6	3		$3\,\Delta G - 2\,\Delta q$
7	4		$4\,\Delta G - 3\,\Delta q$
8	5		$5\,\Delta G - 5\,\Delta q$

Figure 7. The binding of five molecules of GM_1 oligosaccharide to pentavalent cholera toxin occurs with enthalpically enhanced binding; that is, the binding of one GM_1 oligosaccharide increases the favorable enthalpy of binding of the next GM_1 oligosaccharide.[268] Since the individual molecules of GM_1 oligosaccharide translate and rotate independently, the binding in this system is entropically neutral. l = number of ligands, j = number of contacts between GM_1 units, q = interaction energy (enthalpy) of two GM_1 units in contact.

the ΔG of association can be attributed to differences in entropy between these two types of association: The affinity of a polyvalent receptor can be greatly enhanced through multipoint attachment. Because the affinity of the pentamer for the polyvalent surface was not quantified, the extent of cooperativity, if any, is unknown.

The following are two examples of interactions that are probably negatively cooperative (interfering; $\alpha < 1$); that is, unlike the case of positive cooperativity (hemoglobin and O_2), the binding of the second ligand to the second receptor occurs with a less favorable free energy than the binding of the first ligand to the first receptor. The first example is the binding of a bivalent antibody to ligands that are densely packed on a biological surface—such as a mammalian cell, a virus, or a solid support for an enzyme-linked immunosorbant assay (ELISA)—or immobilized in a polymeric matrix (Figure 8).[276] In general, monovalent binding constants of antibodies for small organic ligands vary significantly, but are in the range of $10^5 - 10^8\,\text{M}^{-1}$. For non- or positively cooperative systems, we expect $K_2^{bi} \geq (K^{mono})^2 \approx (10^5 - 10^8)^2\,\text{M}^{-1}$. Karush et al. found that a bivalent antibody for a surface antigen on *Bacillus sp.* bound with 30-fold higher affinity than did the corresponding monovalent antibody (a monovalent antibody is one in which the two or more binding sites present in the native protein

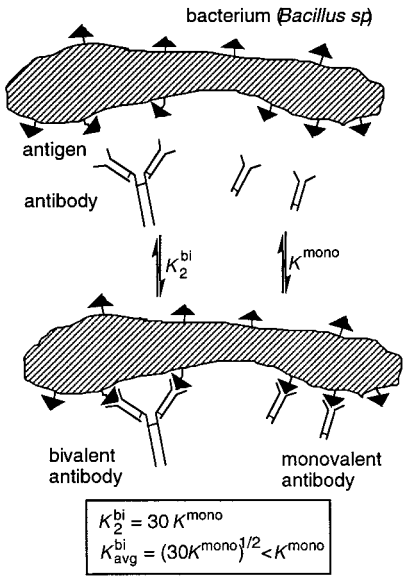


Figure 8. The binding of a bivalent antibody to ligands packed on the surface of a bacterium is negatively cooperative.[270]

have been disconnected chemically or enzymatically); that is, $K_2^{bi} = 30\,K^{mono} < (K^{mono})^2\,\text{M}^{-1}$.[277] The binding here is therefore negatively cooperative.

In a second example (Figure 9) Lee et al. studied the binding of di- and trivalent galactose-containing ligands which bind C-type lectins on the surface of hepatocytes;[206] the

Oligosaccharide	K for complex of lectin/Gal-oligosaccharide (M^{-1})
Gal(β1)OMe	$K^{mono} = 7 \times 10^4$
Gal(β1,4)GlcNAc(β1,2) Gal(β1,4)GlcNAc(β1,4) Man	$K_2^{bi} = 3 \times 10^7$
Gal(β1,4)GlcNAc(β1,2)Man(α1,6) Gal(β1,4)GlcNAc(β1,2)Man(α1,3) Man Gal(β1,4)GlcNAc(β1,4)	$K_3^{tri} = 2 \times 10^8$

Figure 9. Binding of mono-, bi-, and trivalent Gal-terminated oligosaccharides to C-type mammalian hepatic lectins.[201]

density of these receptors is unknown. Where $K^{mono} = 7 \times 10^4\,\text{M}^{-1}$, $K_2^{bi} = 3 \times 10^7\,\text{M}^{-1} = 420\,K^{mono}$, and $K_3^{tri} = 2 \times 10^8 = 2800\,K^{mono}$. Since $K_2^{bi} < (K^{mono})^2$ and $K_3^{tri} < (K^{mono})^3$, these di- and trivalent ligands also bind with negative cooperativity.

These examples illustrate an important characteristic of polyvalent interactions: Even though bivalent binding in these cases was negatively cooperative ($\alpha > 1$), the measured affinity for a bivalent molecule was much higher than for the monovalent molecule. Tight binding does not require positive cooperativity in the sense that this phrase is traditionally used. In the classic study of hemoglobin binding four molecules of oxygen, for example, the free energy of binding of oxygen to tetrameric hemoglobin correlates positively with the number of oxygen molecules bound (see above). In polyvalent bind-

ing, the free energy of binding of each ligand in a polyvalent complex does not have to correlate positively with the number of ligand molecules bound.

Thus, cooperativity, as defined in the traditional biochemical sense, is neither as useful nor as descriptive a parameter for polyvalent systems as it is for monovalent ones. We therefore introduce here a new, empirical metric describing the enhancement of binding of polyvalent systems. By extension from the cooperativity parameter α, we call this new metric β. Thus, although a polyvalent interaction may be qualitatively much stronger than any one of the monomeric interactions contributing to it, these monomeric interactions may still be interfering with,[1] or indifferent to, one another (the "sticking" of polyvalent ligands to affinity gels during affinity chromatography probably represents such a case). Only by a quantitative comparison of polyvalent and monovalent interactions is it possible to establish the nature of cooperativity.

3.3. Enhanced Affinity in Polyvalent Interactions: The Magnitude of β

In many polyvalent systems, the number N of ligand–receptor interactions is unknown. For example, we recently synthesized polymeric, polyvalent inhibitors of agglutination of erythrocytes by influenza virus. These inhibitors were composed of a polyacrylamide backbone, and a certain fraction of the side chains terminated in sialic acid (SA) groups.[2] The SA moieties on this polymer interact specifically with multiple hemagglutinin (HA) receptor sites on the surface of the influenza virus (see Section 2.1) and consequently prevent the interaction of influenza with its target cell. Using a polymer whose side chains present both SA groups and a small number of biotin groups (as a ligand for attachment of enzyme-conjugated streptavidin in a subsequent step in the assay), we measured the binding of this polymeric polyvalent inhibitor to the surface of the virus using an ELISA-like assay (Figure 10).[278] The surface-bound virus was incubated with varying concentrations of polymer containing SA, and the amount of bound polymer was measured indirectly through an enzyme-linked streptavidin–biotin interaction. The measurable quantities in constructing a binding isotherm were the amount of SA contained on polymer bound to the surface of the virus, the concentration of SA [SA], and the corresponding concentration of polymer [P]. The latter was derived by dividing [SA] by the average number Q of SA groups per polymer chain (which was a known quantity in our experiments, and could be calculated from the known degree of polymerization and the experimentally fixed mole fraction of side chains containing SA). Neither the number of polymers bound to the virus nor the number N of SA groups on each polymer that were bound to HA receptor sites were measured. We analyzed this interaction between polymer and virus by determining the amount of bound polymer as a function of [SA]. At half-maximal binding $1/K^{\mathrm{ELISA}} \equiv [\mathrm{SA}] \equiv Q[\mathrm{P}]$, where K^{ELISA} is equivalent to an association constant [Eq. (9), Figure 10]. Since the value of N in this polyvalent interaction is unknown here, no statement

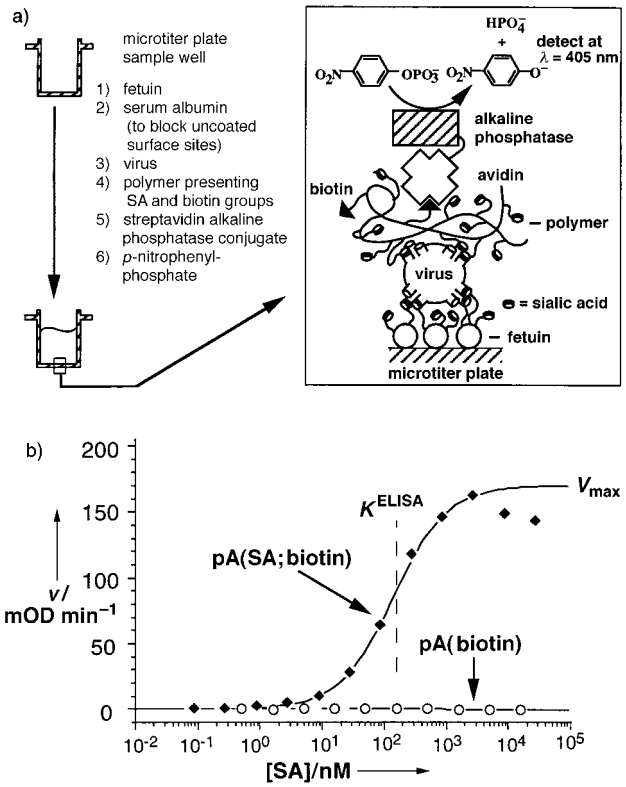


Figure 10. ELISA-type assay for measuring the binding of a biotin-labeled polyacrylamide bearing sialic acid groups (pA(SA; biotin)) to influenza virus. a) Schematic representation of the important binding events occurring during the assay. The influenza virus was immobilized on a fetuin-coated surface from a suspension containing 50 $\mu g\,mL^{-1}$ of viral protein. The polymer (pA(SA; biotin)) was adsorbed to the immobilized virus through SA–HA interactions. The amount of adsorbed polymer was determined by use of a streptavidin–alkaline phosphatase conjugate binding tightly to biotin groups of the polymer: The quantitation was based on measurement of the rate of enzymatic hydrolysis of *p*-nitrophenyl phosphate (given as the rate of change of absorbance at 405 nm). b) A plot showing the binding of pA(SA; biotin) to immobilized influenza virus. The rate of enzymatic hydrolysis was measured as a function of the concentration of polymer-linked SA groups in solution: pA(SA; biotin) The pA(biotin) indicates a polyacrylamide presenting biotin as a side chain: The polymer was tested as a negative control to verify the need for SA groups for binding. The curve superimposed over the data shows a nonlinear fit of the data to a Langmuir isotherm. The constants K^{ELISA} and $v_{\max}$ are shown; $K^{\mathrm{ELISA}} = [\mathrm{SA}]$ at $0.5v_{\max}$. To be consistent in the text, however, we converted K^{ELISA} from [SA] into the equivalent concentration of polymer simply by dividing by the average number of SA moieties on the polymer chain (200). Thus, the converted K^{ELISA} is equal to K_N^{poly}.

regarding cooperativity (α) is possible. The value of K^{mono} in this example is $5 \times 10^2\,\mathrm{M}^{-1}$; the value of K^{ELISA} for our best inhibitor is $10^8\,\mathrm{M}^{-1}$ based on SA; this means $10^{10}\,\mathrm{M}^{-1}$ based on the polymer.[278] That is, monomeric SA binds half-maximally at $[\mathrm{SA}] = 2 \times 10^{-3}\,\mathrm{M}$, whereas the polymer containing SA binds half-maximally to the surface of the virus at $[\mathrm{SA}] = 10^{-8}\,\mathrm{M}$. The polyvalent inhibitor may be therefore useful regardless of its value of α.

We propose an enhancement factor β, which we define as the ratio of the two association constants K^{ELISA} (with respect to the polymer P, and equal to 10^{10} in the example above) and K^{mono} [Eq. (9)], which we prefer to cooperativity (α) in

$$K^{\mathrm{ELISA}} = \beta K^{\mathrm{mono}} \qquad (9)$$

discussion of enhanced affinity of polyvalent systems: Molecules that have high values of β are useful, regardless of whether the interactions that generate them are cooperative or not. In any system where N is unknown, but the total quantity of bound polyvalent molecule is known, β will be a useful parameter. Only if the value of N is known can the precise relationship known between ΔG_N^{poly}, K_N^{poly}, α, and β be calculated [Eqs. (10), (11)]. The quantity β is, using a language familiar to some, the ratio of avidity and the component affinity of the monovalent equivalent to the interaction.

$$\Delta G_N^{poly} = \Delta G^{mono} - RT\ln(\beta) \quad (10)$$

$$\beta = K_N^{poly}/K^{mono} \quad (11)$$

3.4. Enthalpy of a Polyvalent Interaction

The parameter ΔG_N^{poly} is made up of enthalpic (ΔH_N^{poly}) and entropic (ΔS_N^{poly}) components [Eq. (12)]. As a first approximation, the value of ΔH_N^{poly} is the sum of the enthalpies of N mono-

$$\Delta G_N^{poly} = \Delta H_N^{poly} - T\Delta S_N^{poly} \quad (12)$$

valent interactions, $N\Delta H^{mono}$. This value may be made either larger or smaller by other interactions around the active site.

Enthalpically enhanced binding: In some circumstances, the binding of one ligand to a receptor with a given enthalpy may cause the next ligand to bind to its receptor with greater enthalpy; that is, the value of ΔH_{avg}^{poly} is in this case more negative (more favorable) than the value of ΔH^{mono}. Such binding is enthalpically enhanced: One well-studied example (albeit non-polyvalent) is the binding of four molecules of oxygen to hemoglobin. A less well studied (polyvalent) example of enthalpically enhanced binding is pentameric cholera toxin binding to five GM_1 moieties on the surface of a cell (see Section 3.2).

Enthalpically diminished binding: If the binding of one ligand to its receptor interferes with the next binding event, the enthalpy of the polyvalent interaction is less favorable than that expected for N equivalent monovalent interactions. Such binding is enthalpically diminished, and can occur when formation of multiple ligand–receptor interactions between two polyvalent entities requires energetically unfavorable molecular conformations. As a rule of thumb, the more conformationally rigid the polyvalent entity is, the more likely it is that even small spatial mismatches between the ligand and its receptor will result in enthalpically diminished binding (unless the geometric fit between ligand and receptor is accurate at a picometer scale, which is exceedingly rare).

The enthalpy of binding for polyvalent interactions is easy to describe qualitatively, but difficult to estimate quantitatively, either experimentally or theoretically. Consider a bivalent receptor (**1**, Figure 11 a). If the two receptor sites are independent and noninterfering, the binding of two monovalent ligands (**2**) to **1** occurs with twice the enthalpy of binding one ligand ($2\Delta H^{mono}$). For dimeric ligand **3**, in which the two ligands are joined by a rigid group R and fixed

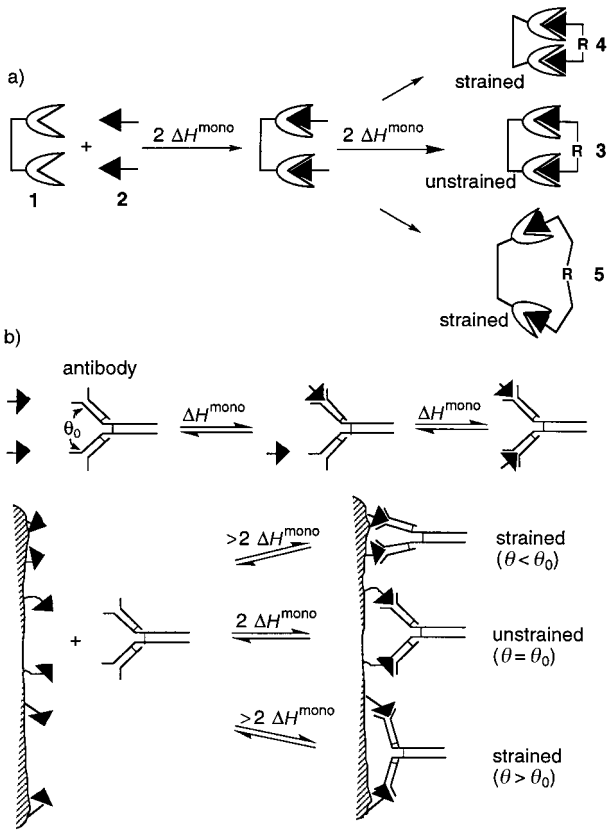


Figure 11. Enthalpy of binding for polyvalent interactions. a) Possible binding modes of monovalent and divalent ligands to a divalent receptor. b) The binding of a divalent antibody to ligand present at variable densities on the surface may be enthalpically diminished due to strain induced by distortion of the antibody from its most stable conformation (θ_0).

at exactly the same geometry as **2**, the enthalpy of binding is twice that of the equivalent monovalent analogue: $\Delta H_2^{bi} = 2\,\Delta H^{mono}$. If the geometry of **3** does not fit the spacing of **1**, the two ligands can still bind, but the aggregate must distort either by displacing the receptors from their equilibrium position to fit the geometry of L-R-L, or by distorting L-R-L from *its* equilibrium position to fit the receptors, or both. Since the average spacing between receptors in proteins is large relative to most organic molecules (for example, the two binding sites in an antibody are approximately 100 Å apart), the most common situation is usually that R is too short (**4**) to allow a good fit of both ligand–receptor pairs. Although it may be possible, using molecular mechanics, to estimate the enthalpic strain introduced in compressing R to fit when it is too long (**5**), it is difficult to estimate the enthalpy of distorting the receptor protein, or of noncovalent interactions between the group R and the protein, or between the subunits of the protein dimer. Figure 11 b illustrates these considerations of enthalpy with an example using the binding of bivalent antibodies to ligands immobilized randomly on a surface.

3.5. Entropy of Interaction

Understanding the entropy of polyvalent interactions is, we believe, essential to understanding the relationship of mono-

valent to polyvalent binding. Incomplete understanding of entropy in the design of polyvalent inhibitors has resulted in many synthetic polyvalent molecules that are less effective or only marginally more effective than their monovalent counterparts. The many bivalent systems joined by flexible linkers (e.g. oligo(ethylene glycol) or polymethylene) provide examples of systems that can almost be guaranteed to fail for entropic reasons.[279, 280]

We can consider the total entropy of a polyvalent interaction ΔS_N^{poly} in terms of contributions from changes in translational ($\Delta S_{trans,N}^{poly}$), rotational ($\Delta S_{rot,N}^{poly}$), and conformational entropies ($\Delta S_{conf,N}^{poly}$) of the receptors and ligands on association, and a contribution accounting for changes in the entropy of the surrounding water ($\Delta S_{H_2O,N}^{poly}$). The latter is often largely due to the entropy of hydrophobic interactions [Eq. (13)].

$$\Delta S_N^{poly} = \Delta S_{trans,N}^{poly} + \Delta S_{rot,N}^{poly} + \Delta S_{conf,N}^{poly} + \Delta S_{H_2O,N}^{poly} \quad (13)$$

3.5.1. Translational and Rotational Entropies

The translational entropy of a molecule arises from its freedom to translate independently through space; the value of ΔS_{trans} is related to the logarithm of its mass M ($\Delta S_{trans} \propto \ln(M)$), and inversely to the logarithm of its concentration ($\Delta S_{trans} \propto \ln([L])^{-1}$). The rotational entropy, ΔS_{rot}, arises from the freedom of the particle to rotate around all three of its principle axes, and is related logarithmically to the product of its three principle moments of inertia I_x, I_y, and I_z ($\Delta S_{rot} \propto \ln(I_x I_y I_z)$). The values of ΔS_{trans} and ΔS_{rot} for a particle are, therefore, only weakly (logarithmically) dependent on its mass and dimensions. To a first approximation, the translational and rotational entropies of *all* particles— ligands, receptors, ligand–receptor aggregates—are equal. When two particles associate, a total of three translational and three rotational degrees of freedom are lost. If the differences in the masses of the particles are ignored (often, the masses of particles are within a factor of 10; almost always, they are within a factor of 100), then the total translational and rotational entropic cost of associating the two particles, whether they be monovalent or polyvalent, is approximately the same provided they are at the same concentration. In biology, concentrations of molecules can vary over more than twelve orders of magnitude (mM to fM). Even though the translational entropy is only logarithmically dependent on concentration, such a wide range makes knowledge of the concentration of interacting particles essential to estimating the importance of translational entropy: This cost increases with decreasing concentration.

3.5.2. Conformational Entropy

Case I: $\Delta S_{conf} = 0$

We discuss the role of entropy in polyvalent interactions using a simple bivalent interaction as a model. The total entropic cost of association of two monovalent receptors with two monovalent ligands is $2\,\Delta S_{trans} + 2\,\Delta S_{rot}$ (Figure 12a). If the two ligands and two receptors are connected by a rigid linking group (that is, one in which no torsional rotation around bonds is possible) having precisely the correct spacing to match the two receptor and ligand sites, then the total entropic cost of assembling these two bivalent species is approximately $\Delta S_{trans} + \Delta S_{rot}$ (half the entropic cost of association of two independent pairs of molecules, and the same as that of one monovalent interaction; Figure 12b). Once a single ligand–receptor interaction occurs between these two rigid bivalent species, subsequent *intra*molecular interactions between the second ligand moiety on the rigid bivalent species and the second receptor site can occur without additional translational and rotational entropic cost, and at no cost in conformational entropy. If there are no additional enthalpic costs, the binding of the second ligand to the second receptor (intramolecularly) occurs with a greater change in free energy ($\Delta H \approx \Delta H^{mono}$, $\Delta S \approx 0$; therefore $\Delta G \approx \Delta H^{mono}$) than does the first. Such binding is entropically enhanced.

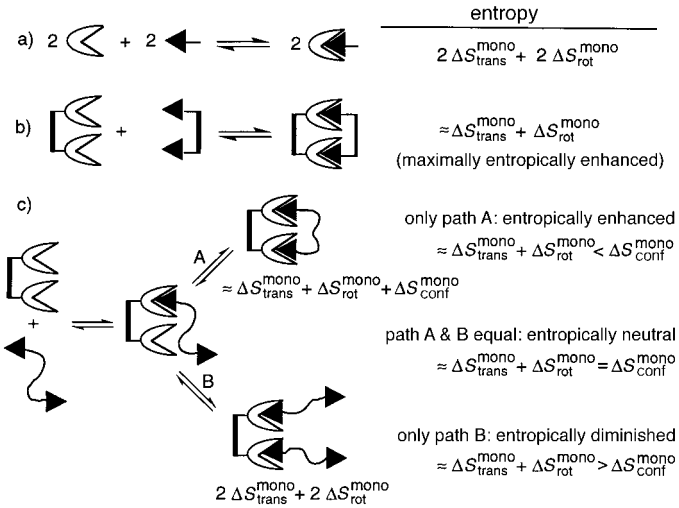


Figure 12. Relationships among translational, rotational, and conformational entropies for a divalent system with a rigid and flexible linking groups.

We have ignored the entropy of hydration thus far, but discuss it in Section 3.5.3. In brief, however, the entropic changes of hydration/dehydration that accompany most biological interactions do not change the spirit of these arguments, and simply add a constant to the value of each entropy discussed.

Case II: $\Delta S_{conf} \neq 0$

Case I is, in general, unrealistic: All linking groups are somewhat flexible, and ΔS_{conf} is almost always less than zero (unfavorable) on complexation; that is, the number of conformations available to the bivalent ligand before complexation is greater than that following complexation. If this conformational cost is less than the total translational and rotational cost, then the total entropic cost of bivalent association is still less than for the monovalent case, and binding is still entropically enhanced (Figure 12c, path A). In the case where this conformational cost equals the translational and rotational cost (Figure 12c, paths A and B), the binding is entropically neutral and the total energetic cost of complexing the second ligand on the dimeric species is the same as the entropic cost of complexing a second dimeric

species. In principle, the conformational entropic cost of complexing the second ligand of the dimeric species can exceed the translational and rotational entropic cost. In that case, the second ligand of the dimeric species will never outdo the binding of a second dimeric species (Figure 12c, path B), and the binding is entropically diminished.

Interestingly, entropy and enthalpy can have partly compensating effects on the affinity of polyvalent interactions: Whereas conformational flexibility increases the conformational entropic cost of association, the same flexibility increases the likelihood that all ligand–receptor interactions can occur without energetic strain. This loss in conformational entropy on association of a polyvalent ligand with a polyvalent receptor has been notoriously difficult to quantitate. The change in entropy on freezing a single, rotating carbon–carbon bond is approximately 0.5 kcal mol^{-1}. The range of values for other single bonds is 0.1–1.0 kcal mol^{-1}.[281, 282] The maximum loss in conformational entropy would occur if all bonds that were initially rotating freely lost all degrees of torsional freedom on complexation. The upper estimate for this loss in ΔS_{conf} is then approximately $0.5\,N$ kcal mol^{-1}, where N is the number of single bonds in the tether linking two ligands or receptors. For a long flexible chain, this number can be large: For a triethyleneglycol spacer, it can be unfavorable to the extent of as much as 10 kcal mol^{-1}.[281, 282]

3.5.3. Entropy of Solvation

A final contribution to the total entropy of association in aqueous systems is the change in the entropy of the surrounding molecules of water, ΔS_{H_2O}. The major contributor to interactions in water (both hydrophobic interactions and those involving polar groups) is the release of organized water from exposed faces of the biological molecules and the resulting increase in entropy. Quantitative measurements and predictions of hydrophobic interactions have been reviewed extensively.[283] These terms will usually have similar values per ligand in monovalent and polyvalent systems (unless the linker changes conformation or associates with the surface of the receptor, and as a result, changes its association with solvent on complexation), and therefore changes none of the arguments presented in the preceding sections. In general, a negative value of ΔS is added to the total ΔS for each ligand–receptor interaction that occurs, irrespective of whether in the context of a monovalent or polyvalent interaction.

There are a number of classes of polyvalent interactions whose entropic characteristics differ markedly. Interactions can occur when both species are initially freely diffusing in solution (the initial six degrees of translational freedom for two such particles are reduced to three degrees following complexation), and we classify these interactions as three-dimensional. When one or both species are restricted to diffusion in a plane or in a line, the interactions are classified as two- and one-dimensional, respectively. The translational and rotational entropic costs of association depend on the number of translational and rotational degrees of freedom lost on complexation. The cost (the combined values of ΔS_{trans} and ΔS_{rot}) is greatest for particles associating in three dimensions, less in two dimensions, and least in one dimension. That is, the entropic cost of association between N ligands freely diffusing in two dimensions (such as ligands tethered to the surface of a cell) with N receptors also freely diffusing in two dimensions (such as trans-membrane proteins on the surface of another cell) is less than for the interaction of these species diffusing independently in three dimensions. We will discuss these issues of dimensionality in Section 4.4, which describes signal transduction by cross-linking of receptors on a surface.

3.6. Kinetics and Enhanced Affinity

Studies of the kinetics for high-affinity polyvalent interactions suggest that the enhancement is mostly due to decreases in the rate of dissociation (k_{off}) of the two polyvalent entities, rather than to increases in the rate of association. Binding of anti-DNP antibodies to DNP-lys, relative to the binding of the same antibody to the DNP-covered surface of ΦX174, established that the values of k_{on} for binding to the surface differed by only a factor of 2 (k_{on}(surface) $\approx 3.7 \times 10^7\,\text{M}^{-1}\,\text{s}^{-1}$, k_{on}(DNP-lys) $\approx 8 \times 10^7\,\text{M}^{-1}\,\text{s}^{-1}$), whereas the values of k_{off} differed by a factor of 10^4 (k_{off}(surface) $\approx 3.3 \times 10^{-4}\,\text{s}^{-1}$, k_{off}(DNP-lys) $\approx 1.0\,\text{s}^{-1}$).[284] Since the rate of a process is related qualitatively (and often quantitatively) to its thermodynamics,[285] these measurements are intuitively consistent with polyvalency: The thermodynamic cost of the first ligand–receptor interaction between two polyvalent entities is approximately the same as the thermodynamic cost of the analogous monovalent interaction; it is therefore plausible that the rates of association might be similar. Dissociation of species interacting polyvalently requires breaking N ligand–receptor interactions; it is therefore plausible that dissociation occurs more slowly in the polyvalent interaction than in the corresponding monovalent one.

4. Characteristics of Polyvalent Interactions in Biology

4.1. Functional advantages of Polyvalent Interactions

In many cases, biological systems seem to use polyvalent interactions rather than an equivalent number of monovalent ones, or one very strong monovalent one, because of certain functional advantages. Examples of functional advantages are discussed in the following.

4.1.1. Achieving Very Tight Binding from Ligands with Modest or Low Surface Area

The magnitude of the total free energy of polyvalent interaction, ΔG_N^{poly}, can be high, and the resulting binding affinities, K_N^{poly}, can be very favorable. In principle, the strength of polyvalent interactions can be much stronger than can be reached by a single interaction between a ligand of low molecular weight and a protein, regardless of cooperativity. The tightest known association for a single interaction between a receptor and a small organic ligand is that between

biotin and streptavidin ($K^{mono} \approx 10^{15}\,M^{-1}$).[286, 287] The polyvalent binding of the most potent, trivalent, naturally occurring oligosaccharides presenting three GalNAc groups to the C-lectin asialoglycoprotein receptor on the surface of hepatocytes occurs with $K_{avg}^{poly} = 10^{8}\,M^{-1}$, even though for simple galactose $K_{mono} = 10^{3}\,M^{-1}$.[206] Interactions with much tighter association constants ($> 10^{15}\,M^{-1}$) have also been claimed.[288] The accuracy in association constants of such magnitude is uncertain as measurement is quite difficult in practice.

4.1.2. Grading Biological Responses or Signaling

In a polyvalent system the strength of a signal can vary greatly, depending on the number of ligand–receptor pairs that participate; to a first approximation, strength might correlate with *N*. This capability to generate a broad range of signal strengths (much broader than the binary "on" and "off" of a single ligand occupying a single receptor site) might, in principle, provide a capability to generate a graded (or graduated) response to a biological signal.

One example of this type of graded response might be the clearance of pathogens by antibody-mediated attachment to macrophages (Figure 13). Where a single antibody is unable to cause a macrophage to ingest a pathogen (macrophages do not bind effectively to a single antibody), two antibodies can lead to ingestion. More antibodies should further strengthen the degree of polyvalency between pathogen and macrophage, and subsequently increase the likelihood that the pathogen will be recognized and cleared. In this case, polyvalency provides a mechanism for recognition: Most pathogens have surfaces that present multiple copies of an epitope. Thus nonspecific adhesion of one receptor on the antibody to a non-polyvalent target on a native (self) surface would not lead to tight, polyvalent adhesion to that surface. Furthermore, macrophages fail to recognize pieces of pathogen if they bind to only a single antibody, but do recognize whole pathogen when it binds to multiple antibodies.[211]

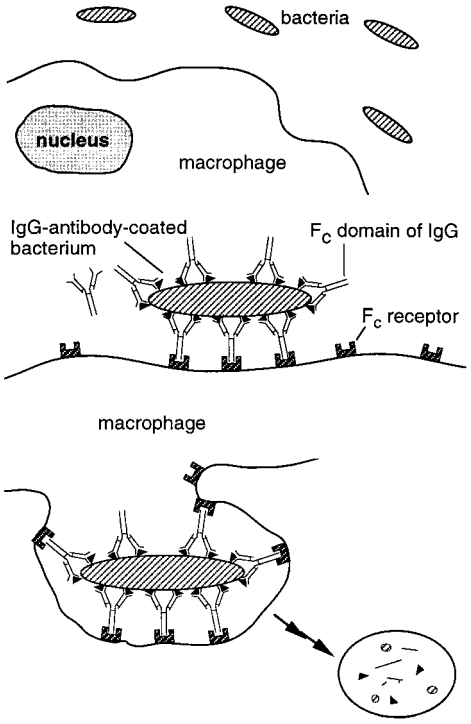


Figure 13. Clearance of pathogens by antibody-mediated attachment to macrophages. An bacterium coated with IgG antibodies is eliminated from the circulation ("cleared") through phagocytosis by a macrophage or neutrophil: The surface of the macrophage has Fc receptors that recognize the Fc region of IgG molecules. The multivalent binding of the antibody-coated bacterium (or virus) to multiple Fc receptors of a macrophage activates the phagocytotic process. Unbound IgG does not bind effectively to the Fc receptors on the macrophage. The representations are not drawn to scale.

Another mechanism by which antibodies lead to the elimination of a pathogen is to trigger the complement cascade (described in more detail in Section 4.1.7; see Figure 16). The constant region on the tail of the antibody, the Fc portion, interacts with one of five clefts on the soluble C1, a pentavalent protein that initiates the complement cascade on binding.[289, 290] Protein C1 is not activated by antibody alone, or by a single antibody–antigen complex. The more densely clustered the antibodies are on a surface (of, for example, a pathogen), the more likely it is that C1 will bind and be activated, and the more likely it is that the pathogen will be destroyed.

4.1.3. Evolutionary Efficiency: Multiplying an Existing Interaction versus Constructing a New One

Biological evolution of new interacting entities may require interactions between new molecules or sets of molecules. Intuitively, there lies evolutionary advantage in borrowing from an existing pool of interactions rather than building new ones. On occasion, environmental forces may confer some evolutionary advantage to an organism that replaces an existing low-affinity interaction with a high-affinity one. Simply increasing the number of individual weak interactions to yield a collectively stronger one replaces the need for evolving a new molecule for a new interaction.

4.1.4. Increasing Strength and Specificity of Binding Through Heteromeric Polyvalency

A polyvalent interaction may involve a mixture of ligand–receptor pairs (more than one type of ligand interacting with more than one type of receptor; we call this type of interaction "heteromeric polyvalency"), and may result in both greater strength and specificity than equivalent monovalent interactions. If, by involving an additional class of ligand, the total number of interactions is increased, then the total strength of the interaction may also increase. The specificity ($A_{N-1}B$ relative to A_N) can increase by differentially regulating the expression of A and B in or on a cell; that is, a cell may bind to another cell expressing only a certain mole fraction of A and B on its surface. Two examples detailed in Section 2 (*E. coli* adhesion and neutrophil adhesion) illustrate heteromeric polyvalency.

4.1.5. Creating Conformal Contact Between Large Biological Surfaces

Figure 14 illustrates an instance where many weak interactions (K_N^{poly}) confer a biological advantage over a single one of equal strength ($K^{mono} = K_N^{poly}$): conformal contact. Such

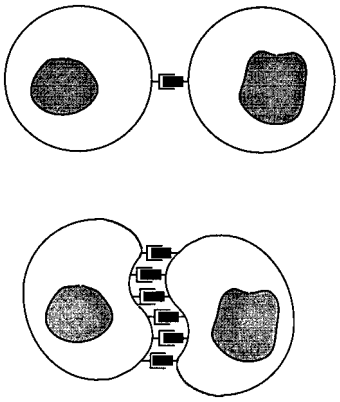


Figure 14. Conformal contact of large biological surfaces (see text for further details).

intimate contact may confer a number of advantages on a pair of cells. For example, the communication between two cells in such intimate contact might be more efficient and more rapid than between cells touching in only one small area. A single strong interaction would not promote intimate, conformal contact between the two surfaces.

4.1.6. Induction of Specific Geometric Shapes and Molecular Distributions

Polyvalent interactions can lead to macroscopic reorganizations and redistributions of molecules. One example of a large change in shape is the conversion of a spherically shaped neutrophil into a flattened one on adhesion to a surface composed of endothelial cells. This occurs after specific interactions between the surfaces of the two cells (Figure 3). An example of a change in molecular distribution is the clustering of receptors in a clathrin-coated pit (Figure 15).

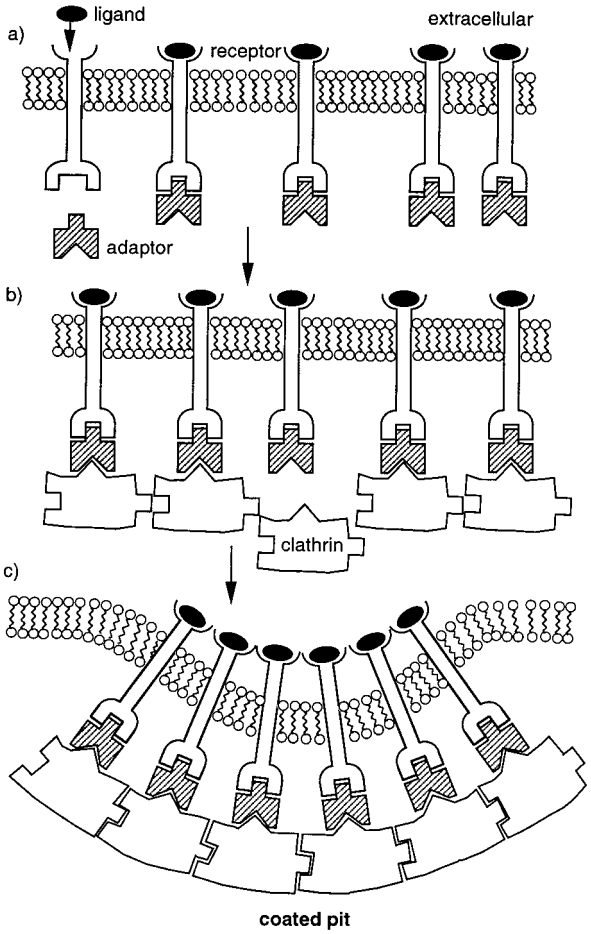


Figure 15. Induction of specific geometric shapes and molecular distributions: clustering of receptors in a clathrin-coated pit. One example is receptor-mediated endocytosis of low-density lipoproteins (LDL). The ligand (LDL) binds to a LDL receptor in the plasma membrane of a cell. Monovalent binding of the ligand to the receptor allows binding of the resulting complex to an adaptor molecule in the cytoplasm, which can induce formation of the clathrin-coated pit by multivalent association of clathrin and other coat-associated proteins.

4.1.7. Signaling by Induction of Large Conformational Changes

In some polyvalent systems, interaction induces a large change in the conformation of one or both of the interacting species. For example, DNA is efficiently stored in a specifically coiled conformation by making multiple, repeated (polyvalent) contacts with a polymeric histone. A remarkable example is that of IgM binding polyvalently to the polyvalent surface of an invading pathogen. Here, a conformation of the IgM that is induced by the polyvalent association serves as a signal. IgM is a class of antibody with five bivalent structures (that is, ten binding sites) extending randomly from a central ring. On binding decavalently to the surface, all five bivalent structures point in a common direction, and portions of the central ring are then more exposed than prior to binding and differently organized. These portions initiate the chain of reactions that constitute the complement cascade (Figure 16) by interaction with soluble pentavalent protein C1 in the blood. This cascade concludes in the assembly of proteins C5b – C9 (the membrane attack complex) in the membrane of the microorganism; this structure causes wide channels (holes) to form in the membrane and results in the death of the microorganism. Binding of IgM to monovalent ligands in the blood does not result in such conformational changes, and does not induce the complement cascade. Polyvalency is necessary in this instance not for tight binding, but for signaling and activation through conformational change.

4.1.8. Preventing Undesired Interaction: Natural Polyvalent Inhibitors

Whereas polyvalency can strongly promote desired interactions, it can also be a useful strategy in preventing certain undesired biological interactions, especially those that are themselves polyvalent (here, polyvalency is used against polyvalency). For example, the fluid coating the interior of the lungs of most mammals contains several mucins (proteins presenting oligosaccharides terminated in sialic acid). These mucins, especially α_2-macroglobulin, can bind to influenza and other SA-binding viruses, and thereby inhibit their attachment to target cells (Figure 17). As we discuss in some detail in Section 5.2, polyvalency may be used here both for

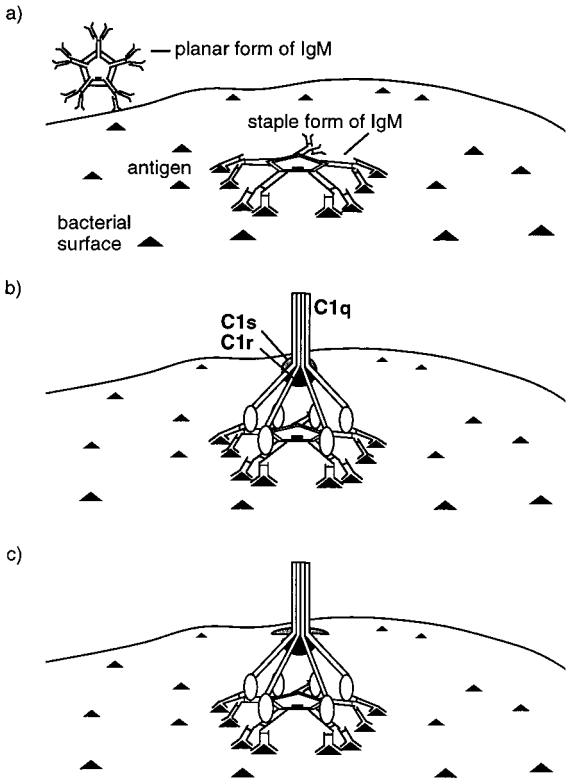


Figure 16. Signaling by induction of large conformational changes. Conformational change of a pentameric immunoglobulin IgM upon multivalent binding to antigens on bacterial surface activates the complement cascade. a) A pentavalent IgM molecule binds to antigens on the surface of the bacterium and adopts a "staple" form. b) C1q (three pairs of Y shape) of the C1 complex (C1q, C1r, C1s) binds directly to a conformationally modified IgM. c) Binding of C1q to IgM leads to activation of the C1 complex (the activation of C1r/C1s—shown as the change in the orientation): C1 is activated only when at least two C1q heads bind simultaneously to antibodies. Such activation of the C1 – IgM complex initiates the complement pathway, leading eventually to the death of a bacterium.

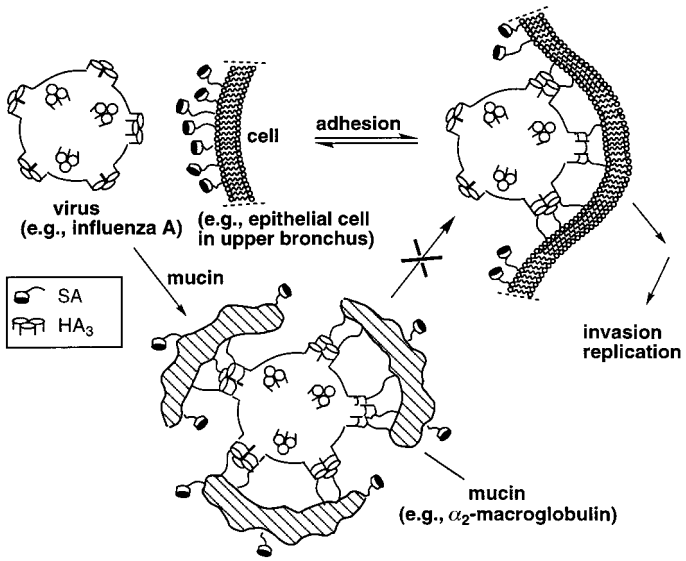


Figure 17. Prevention of the attachment of influenza virus to the surface of a cell. Mucin, a natural glycoprotein with high surface densities of sialic acid groups, is a potent inhibitor of the adhesion of virus by competitive, multivalent binding of the sialyl groups to hemagglutinins on the surface of virus.

tight binding to the pathogen and for steric stabilization of the pathogen surface (a mechanism characteristic of this class of polyvalent interaction).

By rigorously understanding the various systems in which biological organisms use polyvalent interactions at the molecular level, we can begin to see new classes of strategies for pharmaceutical intervention. In the following sections, we discuss well-studied classes of polyvalency, and attempt to illustrate some of the unusual characteristics of polyvalent interactions.

4.2. Attachment of Viruses to Host Cells

Some examples of ligand – receptor interactions important in the attachment of a virus to host cells are given in Table 1.[75] Attachment of a virus to its host is the first step to viral infection, and involves the simultaneous association of multiple molecules on the surface of the virus with multiple molecules on the surface of its host cell (Figure 18).[291] The recognition event between virus and its host cell has been reviewed previously.[75]

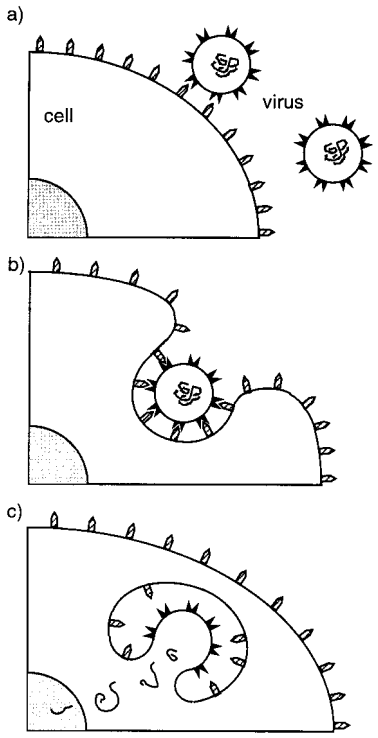


Figure 18. Schematic representation of the entry of viruses into cellular hosts. a) A virus attaches to the surface of a cell through polyvalent interactions of viral receptors and cellular ligands. b) The cell endocytoses the virus. c) Acidification of virus-containing vesicles after endocytosis triggers fusion of the vesicle with cell and exposure of viral DNA.

The molecules involved on the surfaces of the virus and cell that mediate attachment are most directly identified when this attachment is inhibited by either a high concentration of corresponding free monomer of that molecule, or by monoclonal antibodies against that molecule. Such a strategy is used often when the ligand is a sugar or small molecule. A less direct characterization of the molecules involved in attach-

ment comes from correlations of mutations (either man-made or natural) that are unable to bind successfully to the target cell; that is, if a mutation of a particular protein eliminates the ability of the virus to attach, it is involved in the attachment either directly or indirectly. This second strategy is often used for receptors or ligands that are proteins. Although a growing number of molecules on surfaces have been implicated in virus–cell recognition, there are often ambiguities. Because viral particles adhere to many substances nonspecifically, it is often difficult to distinguish nonbiospecific from biospecific binding. Some viral particles may enter a host cell by nonspecific transport (the same mechanism that a cell uses to internalize small molecules). Some proteins on the surface of a virus can recognize more than one type of ligand on the cellular surface. Finally, certain viral particles may associate with a protein freely diffusing in solution, which in turn recognizes molecules on the surface of a cell (mediated attachment). Viruses bind to almost all classes of molecules on cellular surfaces: sugars[292] (for example, polyoma[7] and orthomyxoviruses[293] recognize sialyloligosaccharides), phosphatidyl lipids (for example, vesicular stomatitis virus (VSV) recognizes phosphatidylserine and phosphatidylinositol[58]), and proteins (for example, HIV recognizes CD4,[294] human rhinovirus recognizes intercellular adhesion molecule-1, ICAM-1[295]).

The system for viral attachment and inhibition that has been most extensively studied in the context of polyvalency is the orthomyxovirus A(X-31) (an engineered strain of influenza) attaching to the surface of erythrocytes (Figure 1).[1, 2] This attachment occurs through multiple simultaneous interactions between viral HA and cellular SA. Accurate measurements of the affinity between influenza virus and erythrocyte have not been performed so far. The lower limit for K_N^{poly} is, however, estimated to be $10^{13}\,M^{-1}$. Since the association constant between a single molecule of SA and a single HA receptor is $K^{mono} \approx 10^3\,M^{-1}$, the interaction between cell and virus is clearly polyvalent ($\beta \approx 10^8 > 1$). Since the value of N is unknown, we can make no statement regarding cooperativity (α).

Another extensively studied virus–cell interaction is that between the protein gp120 on the human immunodeficiency virus (HIV) and the 60 K glycoprotein CD4 on the surface of the T-cell (Figure 19). High concentrations of solubilized CD4 (CD4 that has been chemically cleaved from the surface of a cell) inhibits attachment. HIV is an example of a virus that can use alternate molecules on the surface of cells: HIV can infect glioma and rhabdosarcoma cells, both of which lack CD4. Solubilized CD4 does not block attachment and subsequent infection of these cell types.[296]

There are many examples of mediated binding of virus to cells. One example involves the hepatitis B virus, which in some cases binds to aggregates of serum albu min; these aggregates in turn bind to receptors for albumin on the surface of hepatocytes.[297] A second well-studied example begins with the bivalent attachment of antivirus antibodies to the surface of the virus (examples include Dengue virus, West Nile virus, and HIV).[298] Fc receptors on the surface of the target cell then interact polyvalently with multiple Fc tails. This mechanism of attachment is the same as that used by macrophages in recognition of foreign pathogens: interestingly, whereas the former leads to infection, the latter leads to clearance.

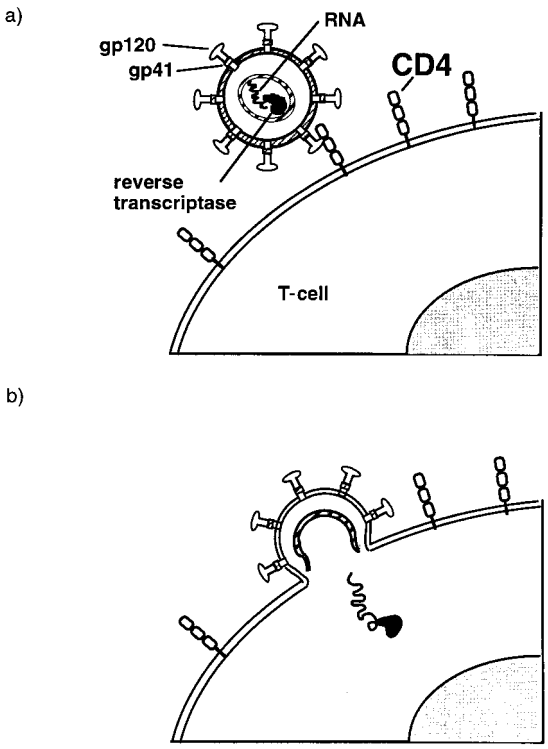


Figure 19. Adhesion of HIV to the target T-cell. a) Binding of gp120 on the surface of the virus to CD4 on the surface of the T-cell. b) Fusion of the virus envelope with the cell membrane and entry of viral RNA genome into the cell.

4.3. Attachment of Bacteria to Host Tissues: Tissue Tropism

Bacterial pathogens can be divided into two classes: intracellular (those that enter a cell and multiply there) and extracellular (those that live among cells, but not inside them). The mechanism of infection by those that are intracellular is similar to that of viruses: Infection is initiated by attachment of the pathogen to the host cell (Tables 2–4). Bacteria that are extracellular often migrate to and collect in particular tissues. Concentration of bacteria in specific tissues (tissue tropism) can occur by specific interactions between molecules on the surface of the bacteria and either molecules on the surfaces of cells in the preferred tissue, or components of the extracellular matrix characteristic of that tissue. Many specific interactions between bacteria and either host cell or extracellular matrix have been reviewed previously.[299, 300] As with viruses, the specificity of the interaction is usually defined by the molecule that is the best inhibitor of an adhesion or agglutination assay. These assays are most often used when the interaction is between a lectin on the bacterial surface and a sugar on either the host cell or the extracellular matrix (Table 2). At present, bacterial interactions appear more likely than viral ones to involve proteins as both ligand and receptor (Tables 3 and 4). These protein–protein interactions are more difficult to study, mainly because the "monomer" is either not known, not available, or loses its "shape" when removed from the the membrane. As a result, most of the detailed studies of bacterial and viral interactions to date involve lectin–sugar interactions, and not protein–protein ones.

Many examples of bacterial interactions with host cells come from the pioneering work of Duguid et al., who were the first to study the agglutination properties of enteric bacteria systematically.[301] Enteric bacteria are those that take up residence in the gastrointestinal tract of mammals. In most cases, the enteric bacteria express fimbriae of type 1, which bind to mannose on the surface of epithelial cells in the gut (for example, enteric *E. coli*, *Klebsiella pneumonia*, and *Salmonella*). This interaction can be assayed by agglutination of guinea pig erythrocytes (the surface of which is densely coated with mannose residues). A well-studied example is that of the uropathogenic *E. coli* described in Section 1.

Certain other enteric *E. coli* bacteria contain fimbrial hemagglutinins on their surface that bind specifically on the surface of the epithelial cell of various glycoproteins containing sialic acid. A well-studied example of sialic acid recognition occurs on an *E. coli* containing the K99 fimbrial lectin,[302] which recognizes *N*-glycolylneuraminic acid located specifically on NeuAc(α2,3)Gal(β1,4)Glc(β1)-ceramide. Enteric strains of *Vibrio cholera* produce a variety of hemagglutinins.[303] The most prevalent of these hemagglutinins binds to fucose derivatives on the epithelial surface.

The host organism often mounts a relatively nonspecific defense that is also polyvalent against these polyvalent pathogens. One example is the Tamm–Horsfall glycoprotein, the most abundant glycoprotein in human urine. This glycoprotein contains a variable number of N-linked oligomannose units, and binds a wide range of bacteria.[304]

4.4. Attachment of Polyvalent Molecules to Cells

Polyvalent molecules can interact with cells and bacteria and cause responses ranging from growth and differentiation to clearance and death. Some of these responses are only possible if the molecule is polyvalent, and would not occur with even a very tightly binding monovalent molecule. We provide examples for antibody–cell interactions, the attachment of toxins to their target cells, and signal transduction through association of polyvalent molecules (hormones, growth regulators).

Immunoglobulins bind polyvalently to the surfaces of cells. As described earlier, the groundbreaking work of Karush et al. showed that the bivalent form of an antibody binds to biological surfaces more tightly than does the equivalent monovalent form.[305] Karush et al. studied the adhesion of both IgG and IgM to a DNP-derivatized surface of the bacteriophage ΦX174, which is specific for *E. coli*. Using a peptic digest, they converted the bivalent IgG and decavalent IgM into monovalent forms, and compared the association constants with those of the unmodified antibodies. They found a 10^3- and 10^6-fold difference for IgG and IgM ($\beta = 5 \times 10^2$ and 10^5, respectively). Moreover, the measured association constant of decavalent IgM, K_{10}^{deca}, was greater than $10^{11}\,M^{-1}$.

More recently, Karush et al. examined the adhesion of a polyclonal mixture of IgG isolated from the serum of a mouse innoculated with a gram positive bacteria *Bacillus sp*.[277] The surface of *Bacillus sp.* presents a peptidoglycan called murein,[306] which exhibits a high degree of periodicity. The association constant for the bivalent IgG was 30-fold higher ($\beta = 30$) than for the monovalent form derived by reduction with 2-sulfanylethylamine. Examining the kinetics of this interaction, Karush et al. also concluded that the rates of association for the bivalent and monovalent forms were almost the same, but that the rate of dissociation for the bivalent species was 30-fold lower than that of the monovalent one.

Two important advantages of a bivalent antibody over a monovalent one are high affinity (β>1) and recognition. The latter is due to a weak affinity of the bivalent antibody for fragments of bacteria floating free in solution and for non-bacterial surfaces that may have epitopes similar to that on the bacterial surface (it is unlikely that the similar epitope occurs at high density on a non-bacterial surface).

A second example is the binding of the human growth hormone (hGH) to receptors on its target cell (Figure 20). This hormone presents two different protein domains held

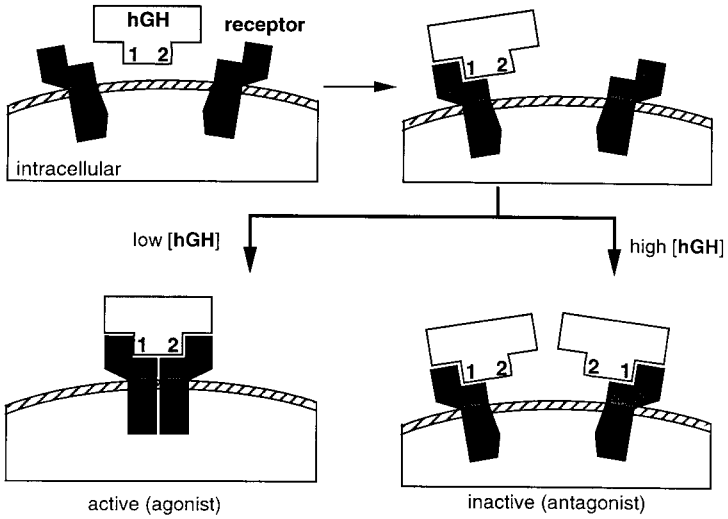


Figure 20. Heteromeric polyvalency. Sequential dimerization model for activation of the human growth hormone (hGH) receptor.

apart by a rigid protein spacer. Although these domains are different, they both recognize the same receptor on the surfaces, but with different affinities. This is another example that falls into the class we previously called heteromeric polyvalency.[307]

4.4.1. Signal Transduction by Cross-Linking of Receptors on a Cellular Surface

Cell growth, differentiation, migration and apoptosis are regulated in part by polypeptide growth factors or cytokines.[308, 309] As these factors are unable to pass the hydrophobic cell membrane, a fundamental question is how they transduce their signal into the cell. A number of ligand–receptor interactions have been identified that involve surface receptor dimerization. Many growth factors and cytokines are polyvalent, and exert their effects through binding and dimerizing (or oligomerizing) cell-surface receptors: Evidence of such a mechanism has been accumulating over the past few years (Table 7), and this area has been reviewed.[308]

Association of a polyvalent ligand to multiple diffusing receptors in a cellular membrane is often involved. In some cases, a single signaling molecule may present two parts, each of which binds a surface receptor (as with human growth hormone, discussed in the preceding section). In this case, the ligand is heterovalent/bivalent. However, polyvalency (as we define it) is not always involved in the mechanism of receptor dimerization. It is entirely possible that in some cases, two ligand-occupied receptors may interact with a favorable energy of dimerization relative to two unoccupied receptors.[257] Dimerization has been observed after binding of a number of cytokines and growth factors: protein–tyrosine kinase receptors, cytokine receptors, antigen receptors, receptors for the tumor necrosis factor (TNF) and related factors, and serine–threonine kinase receptors.[308]

The interaction between an allergen and the surface of a mast cell illustrates some of the unusual properties of systems that use cross-linking of receptors. The mast cell presents multiple copies of the bivalent antibody IgE, and can degranulate (release histamine, which promotes local inflammation) in response to a wide range of allergens. Monovalent ligands for IgE do not trigger degranulation; polyvalent ligands do (Figure 21). The polyvalent binding to the surface of the mast cell can lead to the cross-linking of two molecules of IgE. The cross-linked surface-bound IgE binds (through a transmembrane portion) to an unidentified intracellular protein, and this interaction, in turn, initiates a signal cascade that ends in degranulation. For mast cells, the minimum cross-linking unit needed for release of histamine is two linked IgE molecules,[230, 310] and thus degranulation requires that antigens have $N \geq 2$.[311] Interactions of symmetric and asymmetric bivalent ligands with bivalent antibodies on the surface of the mast cell have been correlated with histamine release.[312, 313]

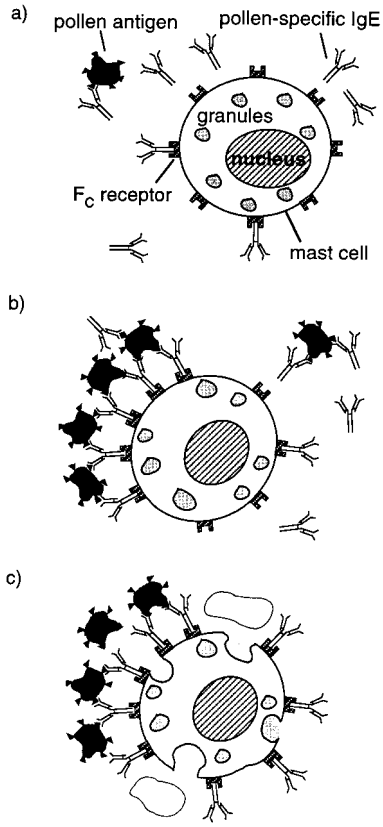


Figure 21. Degranulation of mast cells after binding of a polyvalent allergen. a) After the first exposure to pollen, IL-4 drives B-cells to produce IgE in response to pollen antigens; pollen-specific IgE binds to Fc receptors on the surface of a mast cell. b) The second exposure to pollen leads to the activation of a mast cell by multivalent binding and cross-linking of IgE molecules and antigens. c) An activated mast cell releases its contents by exocytosis of granules containing histamine and heparin, causing allergic rhinitis (hay fever).

Degranulation illustrates an important property of polyvalent interactions in membranes: the receptors in membrane sometimes act polyvalently (for example, by binding polyvalently to the polyvalent allergen) and sometimes not (without the allergen, the receptors do not bind polyvalently to the intracellular protein(s) in the signal transduction cascade). Whether or not receptors bind polyvalently to a polyvalent ligand depends on the detailed balance of three thermodynamic parameters: 1) the enthalpy of complexation; 2) the conformational entropic cost to the ligand on complexation; and 3) the concentration of the receptor in the membrane (which is related to the translational entropic cost of complexation). We have already discussed the first two factors.

The concentration of the receptor in the membrane partly determines the likelihood of signal transduction. The mechanisms by which a cross-linked state of a receptor induces a response in the cell fall conceptually into two classes:

1) An intracellular protein (often a kinase, as for signal transduction following recognition of polyvalent antigen by T-cell receptors[314]) may bind tightly to an intracellular portion made up of portions of both species in the dimer, but not bind, or weakly bind, individual receptors.
2) The two receptors that make up the dimer may chemically modify one another. An example of this is autophosphorylation,[315] in which each receptor phosphorylates the other at specific positions. The phosphorylated species is then recognized by an intracellular protein, such as proteins containing a Src-homology 2 (SH2) domain.[315]

With either mechanism, the step that limits the rate of signal transduction is the ligand-mediated receptor dimerization, and the rate of this step is inversely dependent on the concentration of receptor in the membrane. The translational entropy of a molecule (receptor) in a membrane (two-dimensional) is less than the translational entropy of that molecule in solution (three-dimensional). The binding of a polyvalent molecule to N receptors on a cellular surface can, therefore, be more favorable than the corresponding N monovalent interactions in solution ($-\Delta G_N^{\mathrm{poly}} > -N\Delta G^{\mathrm{mono}}$). The translational entropy of a molecule both in solution and in a membrane is inversely dependent on the concentration of that molecule: the greater the concentration of the receptor in the membrane, the less the entropic cost of complexing a polyvalent ligand. The sensitivity of the cross-linking reaction to the concentration of a ligand is important to both the character and sensitivity of the biological response. The likelihood of a biological response may be regulated by controlling the concentration of receptor or ligand in a membrane, and is the consequence of a balance of two effects:

1) *False signaling*: A spontaneous (unsignaled) cross-link corresponds to false signaling; that is, signaling in the absence of a bivalent ligand. The likelihood of a sponta-

neously formed cross-linked dimer is proportional to the square of the concentration of the receptor in the membrane. The concentration in the membrane must therefore be sufficiently low that the probability of spontaneous cross-linking is acceptably low.

2) *Sensitivity to true signaling*: The translational entropic cost of cross-linking increases with the logarithm of the concentration of receptor in the membrane. The concentration in the membrane must be sufficiently high that the polyvalent allergen is able to cross-link the receptors. An excellent example of the sensitivity of the likelihood of signaling to the concentration of receptor in the membrane is provided by Shur et al.:[253] Sperm expresses molecules of galactosyltransferase (GalTase) receptor on its surface, and these interact specifically with *N*-acetylglucosamine (GlcNAc) groups on the glycoprotein ZP3 on the surface of an egg. Upon productive interaction between sperm and egg, the sperm undergoes a violent morphological change called the acrosomal reaction; this reaction releases enzymes involved in penetration of the protective coating of the egg. Increasing the expression of GalTase on sperm head increases the sensitivity to the ZP3-induced acrosomal reaction.[253]

We put forward the hypothesis that the concentration of receptors that are involved in signal transduction through cross-linking must be under tight control: Raising the concentration would increase the sensitivity of the cell to polyvalent signal, and would also increase the chances of spontaneous, or false, signal transduction; decreasing the concentration would decrease the sensitivity of the cell, and increase the chances of not transducing a signal even in the presence of physiologically effective concentrations of polyvalent ligand.

The dimer that is formed through interaction with a polyvalent molecule in solution may be a homodimer or a heterodimer. The qualitative arguments made above apply in either case. An example of heterodimerization is the binding of the platelet-derived growth factor (PDGF) to the cell surface. The PDGF-A chain binds only α receptors, while the PDGF-B chain binds both α and β receptors. PDGF is a dimer joined covalently through a disulfide linkage. The AA dimer induces the $\alpha\alpha$ receptor homodimer, the AB dimer induces the $\alpha\beta$ receptor heterodimer, and the BB dimer induces all three combinations of receptor dimer.[316]

In some cases, the degree of polyvalency of the ligand determines the fate of the cell with which it interacts. One example is the "positive versus negative selection" of T-cells in the thymus during early maturation of these cells.[317] Direct cross-linking of the TCR by antibodies against CD3 induces conversion of immature CD4+:CD8+ cells into mature CD4+ cells, even in the absence of MHC. In the thymus, peptides that have a high affinity for the MHC sites on the endothelial cells bind to a large number of MHC receptors, and, together with the MHC, present a high-valency surface that interacts with passing immature CD4+:CD8+ T-cells. Peptides that have low affinity for the MHC, however, present a low valency surface for the passing T-cells. Low-valency aggregation appears to be a critical parameter in positive selection leading to mature T-cells. High-valency aggregation appears to be important in negative selection, and leads to T-cell death due to extensive aggregation of T-cell receptors at the interface between the surfaces of the T-cell and the thymic endothelial cell.

Many ligand–receptor interactions are not polyvalent and do not involve receptor dimerization. For such receptors, occupation of the binding cleft by the ligand may induce a specific conformational change that transduces a signal into the cellular interior. One example that involves the integrin family of receptors illustrates the efficiency and versatility of biological systems: Both receptor occupancy and receptor dimerization are important mechanisms on the same receptor.[318] Integrin receptors mediate cell adhesion, signal transduction, and cytoskeletal organization. In one study, receptor occupancy by a monovalent ligand induced receptor redistribution, but minimal signaling through tyrosine phosphorylation. Aggregation by a bivalent noninhibitory antibody (antibody that does not block the cleft on the integrin receptor that binds its ligand) immobilized on the surface of beads induced intracellular accumulation of $pp125^{FAK}$ and tensin, but no accumulation of other cytoskeletal proteins such as talin. Combining monovalent ligand with the antibody induced accumulation of seven cytoskeletal proteins—including α-actinin, talin, and F-actin—thereby mimicking multivalent interactions with fibronectin or polyvalent peptide (natural, polyvalent ligands for this integrin). Integrins therefore mediate a complex repertoire of functions through distinct effects on receptor occupancy or aggregation, or both.

4.5. Attachment of Polyvalent Molecules to Other Polyvalent Molecules: The Control of Transcription

Gene expression is often regulated at the level of transcription. Genes are transcribed when the necessary protein machinery is recruited (that is, associates in a functional conformation) to a region of the DNA near the beginning of the gene. Such recruitment must be sequence-specific. In most organisms, RNA polymerase is recruited by a protein or proteins, transcription factor(s), that bind both to a specific site on DNA and to the RNA polymerase. Very often, the site on the DNA that recognizes the transcription factor is found in multiple copies (from pairs to octets). Also very often, the transcription factors are themselves polyvalent. Bivalent transcription factors (for example a leucine zipper, Figure 22) are found in many forms: Some are homodimers, and others are heterodimers; some are joined covalently, and others are joined noncovalently. In almost all cases, transcription factors that bind to the DNA as dimers or multimers do not bind as monomers: polyvalency is used in transcription to increase both the strength and the specificity of attachment; note, for example, the interaction of DNA with retinoid X receptor (RXR) transcription factor (see Section 2). In this example, small changes in concentration of a transcription factor lead to large, nonlinear increases in the rate of transcription through a polyvalent mechanism (the rate of transcription is approximately proportional to the $[RXR]^5$). The presence of more than two half-sites in one hormone response element is not unique to RXR response elements (Table 7).[319]

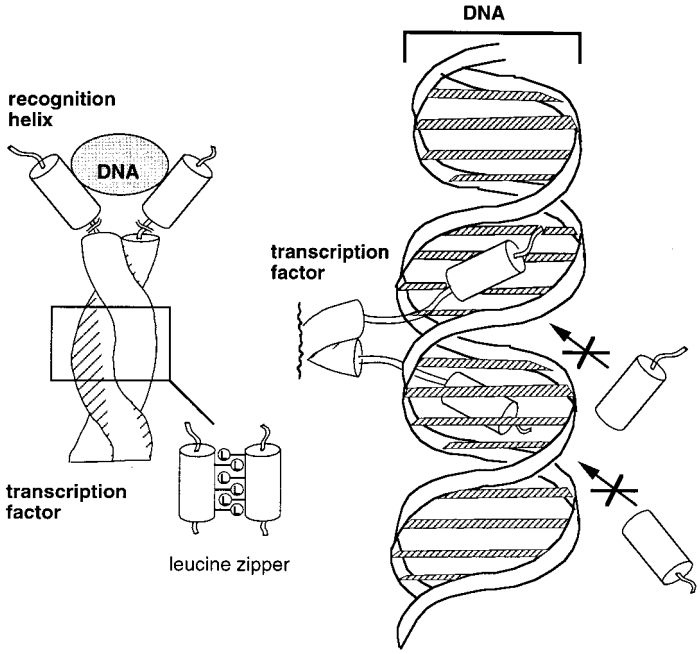


Figure 22. Dimeric transcription factors. A coiled coil of α helices from two separate polypeptide subunits, stabilized by a leucine zipper, holds two recognition helices in a position to bind sequences in the major groove of DNA.

4.6. Attachment of Cells to Other Cells

A number of other cell–cell interactions may occur polyvalently (Table 5). One example described in Section 2.3 is neutrophil attachment to endothelial cells near a site of tissue injury. A second example may be the clearance of non-sialylated cells from the blood by the liver (Figure 23). Lee

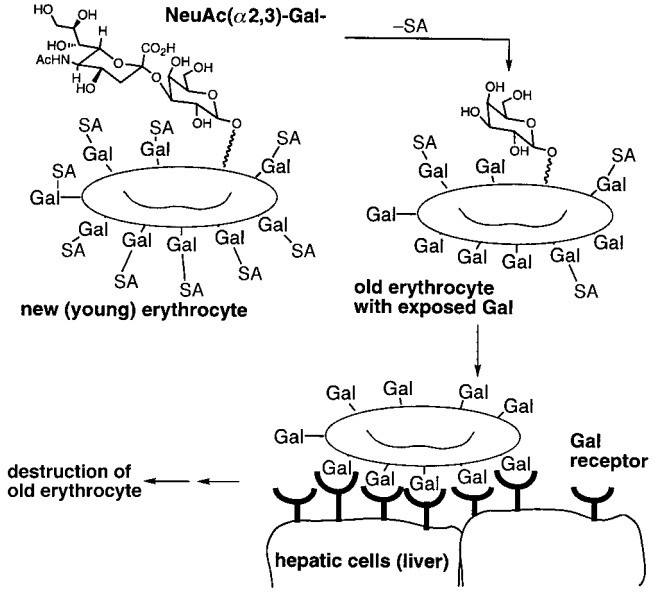


Figure 23. Clearance of non-sialylated erythrocytes from the blood by liver.

et al. have examined extensively the role of polyvalency in the association of synthetic di- and trivalent galactose-containing ligands to C-type lectins on the surface of hepatocytes.[206] An example of a cell that becomes progressively desialylated, as a reflection of age, is the red blood cell (erythrocyte). Desialylation exposes a galactosyl group, and occurs as a consequence of the hydrolytic action of different neuraminidases, both free in the blood and bound to endothelial cells that line the interior of the blood vessels. The probability of hepatic clearance may be correlated with the interaction energy between the surfaces of the hepatic cell and the erythrocyte. As the density of galactosyl ligands on the surface of the erythrocyte increase, the interaction energy also increases. Thus as red blood cells age, the density of the "clearance signal" on their surface increases until the interaction energy between its surface and that of the hepatocyte is sufficiently high that it promotes adhesion and clearance. The resulting lifetime of a typical red blood cell in human blood circulation is 120 days. We suggest that polyvalency may, in this instance, be used as part of a timer that enables the body to judge the age of erythrocytes and select those that are old enough to be removed from circulation and destroyed.

A third example of cell–cell interactions is that between a class I MHC-bearing antigen-presenting cell and a CD8+ T-cell; both are essential components of the immune system (Figure 24). The cell presenting antigen may, for example, be a

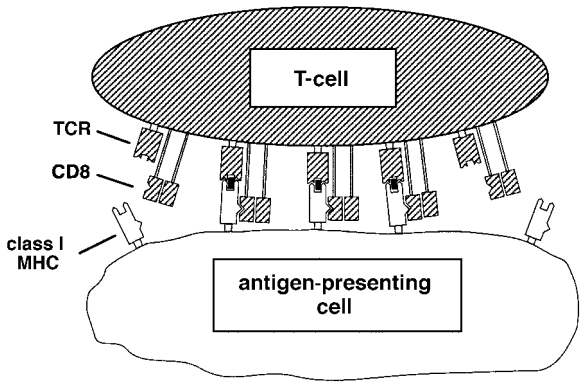


Figure 24. Interactions between a cell (bearing class I MHC and presenting antigen) and a T-cell (expressing T-cell receptor and CD8). Class I MHC interacts with both CD8 and the TCR.

cell infected by a virus: Some small but finite portion of the proteinaceous components of the virus produced by the cell are degraded intracellularly into small peptides composed of nine amino acids. These nonapeptides are then presented on the surface of the infected cell as an extracellular binding cleft of a class of transmembrane protein called the class I major histocompatibility complex (MHC).[320] This complex is recognized by a complementary T-cell receptor on the surface of a CD8-bearing (cytotoxic) T-cell. The consequence of one such recognition event is unclear. The consequence of several such events is the transduction of a signal into the interior of the T-cell that induces it to kill the cell that is presenting the antigens. The requirement for a large number of discrete recognition events prevents excessive destruction of cells that are not infected; that is, a high interaction energy distributed across multiple interactions corresponds to a high level of certainty that the cell harbors a virus inside.

A fourth example of cell–cell interactions is that involved in cell migration. A well-studied interaction that may be important in cell migration is that between the galactosyltransferase (GalTase) protein on one cell and the GlcNAc-terminated glycoproteins and glycolipids on another cell. Cells express different densities of both GlcNAc and GalTase. The levels of expression may vary with time and biological environment. The strength of adhesion between the cells is related to the number of contacts between the two surfaces, and therefore to the densities of both GlcNAc and GalTase. In a classic experiment, Schnaar et al. showed that a malignant cell expressing GalTase on its surface will migrate along a concentration gradient of immobilized GlcNAc, whereas the same cell not expressing GalTase will not.[341] Shur et al. have demonstrated that cells expressing GlcNAc will migrate along a concentration gradient of immobilized GalTase.[204] This interaction between GalTase and GlcNAc may be important in a range of cell–cell interactions—including those necessary for a metastatic tumor to attach to, or migrate to, a remote site (Figure 25)—and for productive interaction of a sperm with an unfertilized egg.[318]

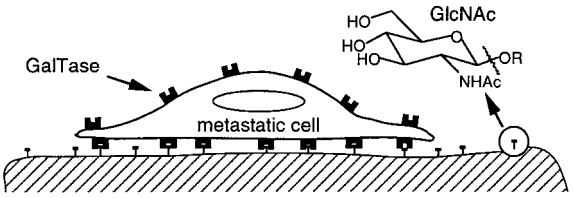


Figure 25. Adhesion of a metastatic cell to laminin. GalTase is expressed on the edges of migrating cells, where it is associated with the cytoskeleton and mediates cell migration by binding to oligosaccharides terminated with *N*-acetylglucosamine (GlcNAc).

5. Implications for Medicinal Chemistry: Polyvalent Pharmaceuticals

Polyvalency is ubiquitous in biology. An understanding on the molecular level of biological systems that are polyvalent suggests strategies for the design of drugs. In principle, polyvalent molecules might both agonize and antagonize; experiments to date have been designed to inhibit undesired biological processes (antagonize) rather than to promote desired ones (agonize).

5.1. Polyvalent Inhibition

A polyvalent inhibitor has two or more linked ligands that act simultaneously at two or more linked receptors. The collection of ligands can be joined covalently (joined through a defined linker,[311, 313, 321–328] tethered to the backbone of a polymer,[2, 329, 330] tethered to the head groups of a dendrimer,[331] or tethered to the surface of a small protein), or noncovalently (tethered to the head groups of molecules in liposomes, membranes, or surfaces[194, 332, 333]). Examples of the various backbones that have been successfully used to construct polymeric inhibitors are given in Table 9. Each has a different advantage and disadvantage with respect to ease of synthesis, flexibility with which it can be modified synthetically, biodegradability, bioavailability, and toxicity. As an example of an advantage, polyacrylamides of high molecular weight are more effective inhibitors of hemagglutination induced by influenza virus than are polyacrylamides of low molecular weight;[278] as examples of disadvantages, these polyacrylamides of high molecular weight are also toxic, less easily cleared by the kidney, and more difficult to synthesize.

One issue that may limit the use of certain polymers of extremely high molecular weight, including polyacrylamides, is that molecules of size greater than that of a protein of 60–70 kDa cannot be filtered effectively by the kidney (mainly because such molecules are too large to pass through the pores in the glomeruli of the kidney). Such large molecules must be degraded enzymatically in the liver (degradative clearance), and can thereby produce a wide range of metabolites that are potentially toxic. One strategy would be to join small polyvalent inhibitors through biodegradable linkages. The rate of hydrolysis of these linkages must be greater than the rate of uptake of these polymers into the liver. There are expected to be, however, certain polymeric structures of high molecular weight that are effectively benign in vivo.

Polyvalent inhibitors that bind the pathogenic particle have advantages over inhibitors that bind the host. One may, in principle, use either partner of a ligand–receptor pair as the basis for a polyvalent drug to inhibit their interaction. Generally, we expect that a strategy that involves the binding of pathogen will cause fewer side effects than the binding of the molecules characteristic of the host. The choice in the cases of non-infectious diseases may be less clear because both partners in the ligand–receptor pair may be natural to the patient.

5.2. Mechanism of Polyvalent Inhibition

5.2.1. Inhibiting Attachment of a Virus to a Host Cell

We[1, 2] and others (Matrosovich et al.,[329, 356] Roy et al.,[357, 358] Gamian et al.,[359] and Sabesan et al.[360]) have constructed a wide range of polymers that inhibit the attachment of influenza virus to erythrocytes (which serve as surrogate target cells). This system is currently the most extensively studied of all polyvalent interactions, and serves as the prototypical system on which many of our conclusions are based. The most effective class of inhibitors to date is the linear polymer polyacrylamide presenting multiple copies of the C-glycoside of sialic acid (SA) as side chains (Figure 26): The most effective of this class prevents hemagglutination (the cross-linking of erythrocytes by virus) at concentrations of 35 pM, whereas the monomeric α-methylsialoside inhibits hemagglutination at concentrations of greater than 2 mM (for details of this SA–HA interaction, see Section 4.2).[2, 335] This 10^8-fold enhancement in ability to hemagglutinate erythrocytes is the largest known increase in activity for any synthetic polyvalent system known to us. Since we do not know the number (N) of attachment points between the polymer and the viral surface, we can make no statement regarding the degree of cooperativity (α) of binding [Eq. (8)]. Regardless of the extent of cooperativity, however, the polyvalent inhibitors are remarkably effective and may illus-

Table 9. Examples of synthetic multivalent ligands.

Multivalent ligand	Multivalent receptor	Assay	Enhancement of binding (β)
polyacrylamide with SA-containing side chains prepared by copolymerization[334]	HA on the surface of influenza virus X31	HAI[a]	$10^3 - 10^4$
polyacrylamide with SA-containing side chains prepared from pNAS[2, 335]	HA on the surface of influenza virus X31	HAI, OPTCOL	$10^5 - 10^8$
polyacrylamide with SA-containing side chains prepared from pNAS[278]	HA on the surface of influenza virus X31	ELISA-binding assay	$10^4 - 10^5$
poly(7-oxanorbornene) containing α-mannose as side chains,[336] prepared by ROMP[b]	concanavalin A	cell agglutination assay mediated by concanavalin A	5×10^4 based on α-mannose
poly(7-oxanorbornene) containing β-glucose as side chains,[330] prepared by ROMP[b]	concanavalin A	cell agglutination assay mediated by concanavalin A	2×10^3 based on β-glucose
dendrimers containing SA[331, 337]	HA on the surface of influenza virus X31	inhibition of horse radish peroxidase	10^2
dendrimer (Man-α)$_{16}$[337]	concanavalin A	inhibition of binding of concanavalin A to yeast mannan	4
dendrimer (NeuAc-α)$_{15}$[338]	lectin (*L. flavus*)	inhibition of lectin to orosomucoid	12
dendrimer (*N*-acetyllactosamine)$_{2-8}$[339]	erythrina cristagalli lectin	inhibition of the interaction between lectin and porcine from stomach mucin (IC50)	1 – 10
dendrimer (GlcNAc)$_{2-8}$[339]	wheat germ agglutinin	inhibition of the interaction between lectin and porcine from stomach mucin (IC50)	1 – 10
liposomes containing SA[332, 333]	HA on the surface of influenza virus X31	HAI	$10^4 - 10^6$
liposomes containing 332 antibodies (664 binding sites)[340]	modified red blood cells	fluorescence quenching	2 – 10
liposomes containing sLe^X mimetics[194]	P-selectin IgG conjugate	inhibition of binding of P-selectin IgG to HL-60 cell	2×10^6
GalNAc linked to BSA[c] (BSA(GalNAc)$_{39}$)[341]	amebic surface lectin	inhibition of human red cell mediated by *E. histolytica* lectin	1.4×10^5
T-10 fragments (glycopeptide) were polymerized	*M. Prolifera* cells	competition assay using radioactive monomer and cold polymer	1
α-melanotropin (MSH) linked to TMV[d][342]	Cloudman S-91 melanoma cell	agonist activity for tyrosinase stimulation	6
dimeric α-MSH[343]	melanocytes	functional melanocyte dispersion assay	7
liposomes containing enkephalin[344]	bovine brain homogenates	displacement of radioactive (^{3}H-labeled) enkephalin	13
α_2-macroglobulin (4-*O*-Ac-Neu5Ac)[345, 346]	HA on the surface of influenza virus X31	HAI	10^5
mono-, di-, tri-, and tetraantennary galactose derivatives[347]	rabbit hepatocytes[348]	inhibition of radioactive glycopeptides	10^5 (triantennary)
triantennary galactoside[349]	hepatic asialoglycoprotein receptor	in vivo	2×10^3
bivalent SA[279, 280]	influenza virus (hemagglutinin)	binding assay (^{1}H NMR)	10^2
bivalent sLe^X[196]	E-selectin	inhibition of adherence of E-selectin to HL-60 cells	5
bivalent sLe^X *O*-glycan[350]	lymphocyte	inhibition of adherence of lymphocyte to endothelial cell	> 50
bivalent cyclosporin[351]	cyclophilin (Cyp) A	fluorescence titration assay	≈ 1 for monomeric Cyp, > 1 for membrane-bound Cyp
bivalent indole linked by variable length alkanes[352]	serotonin receptors: 5HT$_{1A}$, 5HT$_{1D}$, 5HT-UT, and 5HT$_{1E}$	radioligand displacement assay	4×10^2
bivalent L-Lys-D-Ala- D -Ala linked by a rigid 10-Å linker[353, 354]	synthetically bivalent vancomycin linked by a succinyl linker	competition assay with the monomer using capillary electrophoresis, fluorescence, and surface plasmon resonance assays	10^3
bivalent opiods[355]	guinea pig brain membranes	inhibition of radioactive ethylketazorine	20

[a] Hemagglutination inhibition assay. [b] Ring-opening metathesis polymerization. [c] Bovine serum albumin. [d] Tobacco mosaic virus.

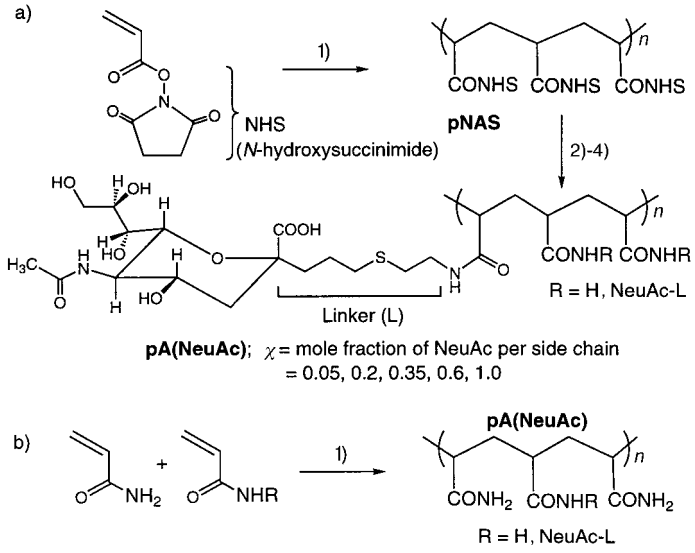


Figure 26. Synthesis of polyacrylamides with side chains containing multiple copies of α-C-sialosides, pA(NeuAc). a) Derivatization of an activated ester of poly(*N*-acryloyloxysuccinimide) (pNAS): 1) radical-catalyzed polymerization; 2,2′-azobisisobutyronitrile (AIBN), tetrahydrofuran (THF), 60 °C; 2) χ molar equivalents of NeuAc-L-NH_2 (per mole of activated ester on the polymer), triethylamine, *N*,*N*-dimethylformamide (DMF), room temperature; 3) excess ammonia; 4) dialysis against distilled water. By varying the number of equivalents of NeuAc-L-NH_2 (χ equiv), five types of pA(NeuAc, $\chi = 0.05$, 0.2, 0.35, 0.6, 1.0) were prepared; χ denotes the mole fraction of NeuAc in the polymers and is defined as the number of side chains containing NeuAc divided by the total number of side chains. b) Direct radical-catalyzed copolymerization of a mixture of two or more monomeric acrylamides.

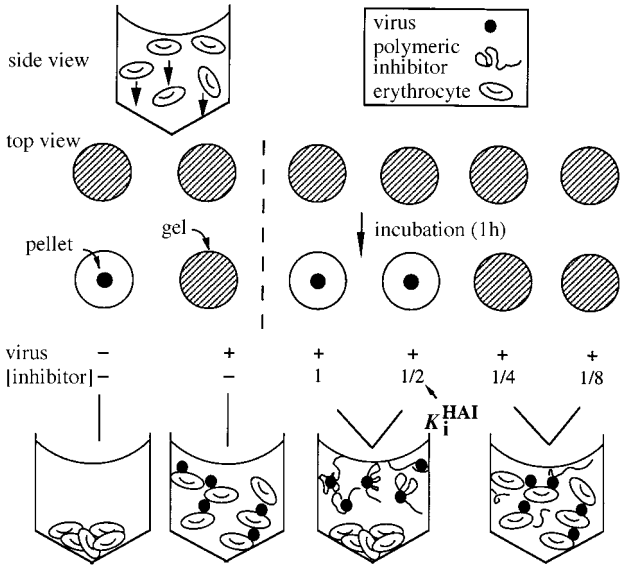


Figure 27. Schematic representation of the hemagglutination inhibition (HAI) assay. The assay requires a sequential mixing of three components in a microtiter well; 1) incubation (30 min) of virus and inhibitor (at various concentrations); 2) addition of erythrocytes to the mixture followed by incubation for about one hour. The 250-μL microtiter well is viewed both from the side and the top. Hemagglutination (formation of a gel) is represented by hashed marks; a pellet of erythrocytes is shown as a dark spot. The concentration of the inhibitor is one unit in the third well from the left, and is sequentially lowered by a factor of two in the wells to the right of the third well. The value for the lowest concentration of inhibitor that inhibits hemagglutination is, in this case, 0.5 units.

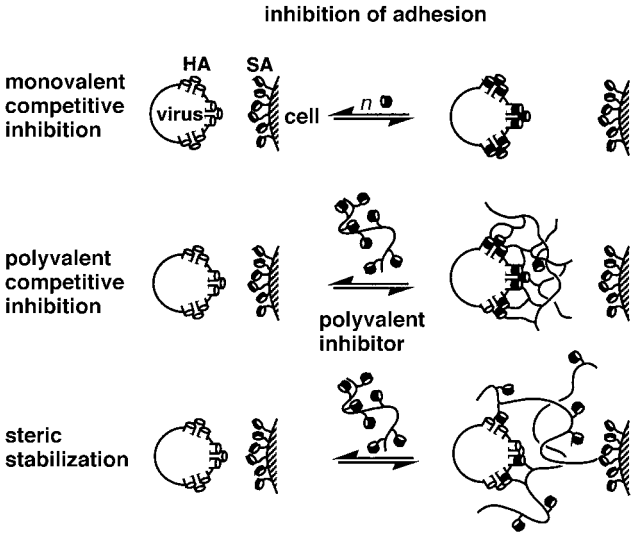


Figure 28. An example of inhibition of an unwanted polyvalent interaction using polyvalent molecules. Influenza virus attaches to its host cell through polyvalent interaction of HA with SA. Monomeric molecules containing SA units inhibit attachment by competitive inhibition, whereas polymeric molecules inhibit attachment by a combination of two conceptually independent, limiting mechanisms: 1) high-affinity binding through polyvalency (polyvalent competitive inhibition) and/or 2) steric stabilization of a surface (steric inhibition).

trate a general strategy for inhibition of interactions between two polyvalent surfaces; the values of β for a number of polymeric inhibitors are much greater than 1, and these classes of inhibitors are therefore potentially very useful.

This system is the one that has been explored in greatest detail to date with respect to mechanism of action of polyvalent inhibitors, and has had the structural determinants of effectiveness most clearly defined. The hemagglutination inhibition (HAI) assay has mainly been used to evaluate polyvalent, polymeric inhibitors of influenza viral attachment. This assay measures the lowest concentration of inhibitor (in terms of sialic acid groups, whether free in solution or bound to a polyvalent carrier) that is able to inhibit the virally mediated cross-linking of erythrocytes (Figure 27).

There are, in principle, two different limiting mechanisms of action for polyvalent inhibition of influenza virus to erythrocytes, the sum of which contribute to the observed inhibition constant K_i^{HAI} (Figure 28): 1) entropically enhanced competitive inhibition and 2) steric stabilization. We have performed experiments that have deconvoluted the results from hemagglutination inhibition into contributions from entropic enhancement of inhibition and steric stabilization, and showed that both mechanisms of action are significant.

5.2.2. Competitive Inhibition

We define this mechanism as that acting through binding of the inhibitor (a structural analogue of sialic acid, SA) at HA, and therefore competitively preventing the binding of cellular SA to the same HA. The ability of an inhibitor containing SA to inhibit an interaction competitively is related directly to its dissociation constant with the viral surface. Figure 29 illustrates three related equilibria and their corresponding association constants: The association constant between mono-

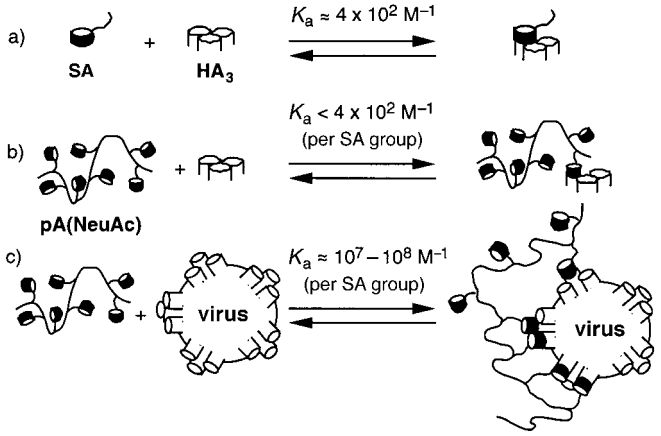


Figure 29. Association constants (K_a) for the association of sialic acid (SA, NeuAc) and hemagglutinin (HA): a) monomeric SA and HA from HA_3; b) SA from polyvalent polyacrylamide, pA(NeuAc), and HA from HA_3; c) SA from polyvalent polyacrylamide, pA(NeuAc), and HA on the surface of virus.

meric SA and trimeric hemagglutinin (HA_3) is $4 \times 10^2\,M^{-1}$ in terms of SA; that between polyacrylamide presenting multiple copies of SA and HA_3 is less than $10^2\,M^{-1}$ (unmeasurably small with NMR and fluorescence assays);[1] and that between SA on a polymer and HA on the viral surface is 10^7–$10^8\,M^{-1}$.[278] A comparison of the magnitudes of these equilibria is useful: The polymer appears to bind more poorly to HA_3 ($N=3$) than does the monomeric SA ($N=1$), in spite of a greater degree of polyvalency. This lower affinity of the polymer for HA_3 may be due to steric interference of the large, water-swollen polymer backbone on the interaction between each SA group and its binding cleft on HA_3. The binding of the polyvalent SA to the polyvalent surface (expressing multiple HA_3 receptors) is, however, much more effective than the binding of polyvalent SA to HA_3. That is, by increasing the degree of polyvalency from $N=3$ to $N \approx 10-10^2$ (the number of HA receptors bound to one polymer molecule), any steric interference in binding has been overwhelmed.

Since the association constant K_a is a direct measure of the ability of an inhibitor to inhibit competitively, a comparison of the values of K_a and $1/K_i^{HAI}$ (K_i^{HAI} has units of a dissociation constant, and $1/K_i^{HAI}$ has units of an association constant) provides a measure of the importance of competitive inhibition in preventing hemagglutination. We have examined the binding of virus to a polyacrylamide in which 20% of the side chains were derivatized with SA; this mixture of polymers were fractionated into portions that varied in molecular mass.[278] We have shown that the values of K_a for these polymers ranged between $10^6-10^8\,M^{-1}$. The values of $1/K_i^{HAI}$, on the other hand, varied between 10^7 and $10^9\,M^{-1}$. That is, the ability of the polyvalent inhibitor to bind tightly to the viral surface accounts for much, but not all, of its enhanced effectiveness over monovalent inhibitors.

5.2.3. Steric Stabilization

This mechanism involves inhibiting the interaction of the viral surface with the cellular surface by a means other than competitively occupying the binding clefts on HA. We envision the polymer forming a loose, water-swollen, gellike layer on the viral surface. This layer sterically prevents the close approach of the cellular surface. An analogous mechanism may play an important role in keeping microparticles stable in suspension (as is often used in the food industry) by the addition of hydrophilic polymers such as poly(ethylene glycol) and poly(acrylic acid). In a limiting case, the polymer presenting SA may bind with very poor affinity, but a single binding event brings the large, water-swollen polymer to the viral surface.

We have performed two sorts of experiments that demonstrate that steric stabilization may be important in inhibiting virus–cell interaction by polymeric polyvalent molecules: The first experiment is indirect, and we describe it below. We concluded that even though the polymer examined was $1/K_i^{HAI} \approx 10^7$ times more effective at preventing hemagglutination than an analogous monomer, the affinity of the polymer for the viral surface was only 10^6 times higher; that is, the additional factor of 10 was likely due to a mechanism other than affinity, and we hypothesize that this additional enhancement is due to steric stabilization.

The second experiment is more direct than the first: We attempted to increase the ability of a given polymer to stabilize the virus sterically, and simultaneously to decrease the affinity of this polymer for the surface of the virus.[361] We first describe the experiment and the results, then we rationalize these results in terms of mechanism. The SA groups on the polymer bind to two sites on the surface of the virus: HA and NA (neuraminidase, a second protein on the surface of the virus that is less abundant than HA). Binding of SA to both sites may be important in determining the affinity of the polymer for the surface of the virus. We synthesized three different monomeric inhibitors of NA that do not bind significantly to HA, and do not inhibit agglutination by itself even at concentrations as high as 40 mM. We found that addition of any of these NA inhibitors augmented the effectiveness of the polymer in inhibiting viral hemagglutination in a concentration-dependent manner (Figure 30) that depended on the affinity of the NA inhibitor for NA. For polymers bearing a high mole fraction of sialic acid, the enhancement was 20-fold in the presence of NA inhibitor; for polymers bearing low mole fractions, the enhancement was twofold or less.

Displacement of a sialic acid on the polymer from a NA site by the NA inhibitor would have two effects (Figure 31): It would lower the affinity of the polymer for the surface of the virus (that is, it would lower the total number of ligand–receptor interactions), and it would increase the size of the loops that extend from the surface of the virus and thereby increase the ability of the polymer to inhibit the approach of another surface (steric stabilization). The NA molecules rise only 50 Å from the viral surface, while the HA molecules rise 100 Å. Release from low-lying NA sites may create a more effective barrier between the surface of the erythrocyte and the SA-binding sites on the tips of the HA. This example demonstrates that steric stabilization is not only an important mechanism of action of polyvalent inhibitors, but also that it can be modulated.

The mechanism of polyvalent inhibition will certainly be dependent on the system, but we have shown through a

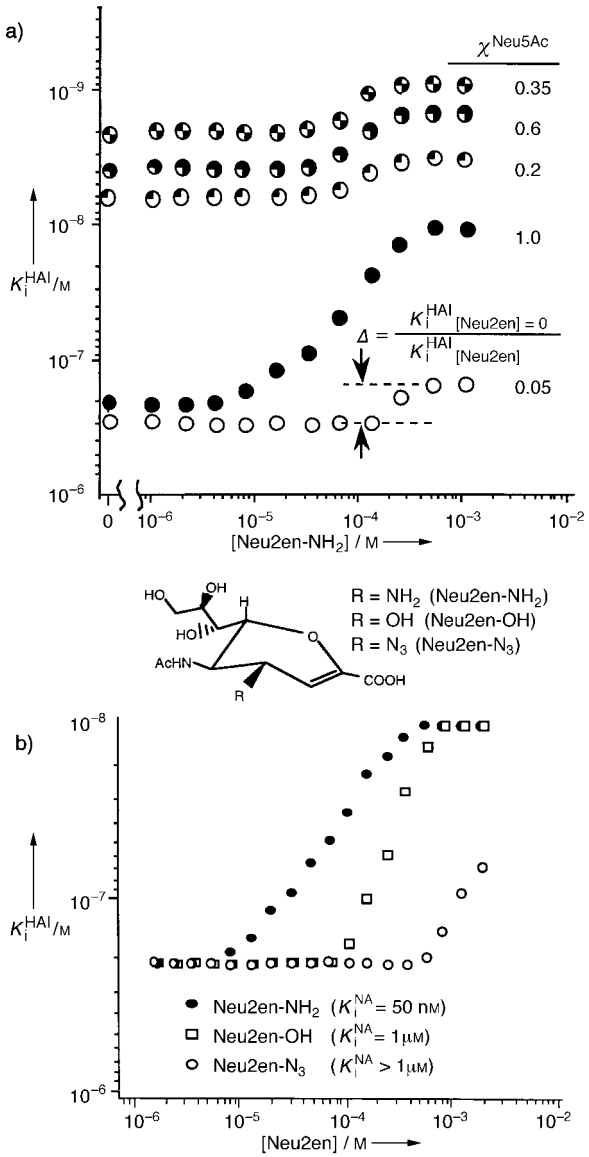


Figure 30. Augmentation of the effectiveness of the polymeric sialic acid inhibitor by monomeric neuraminidase (NA) inhibitors (Neu2en). a) The values of pA(NeuAc) ($\chi^{NeuAc} = 0.05$, 0.2, 0.35, 0.6, 1.0) are shown as a function of the concentration of Neu2en–NH_2 (50 nM – 50 mM). The presence of Neu2en–NH_2 in the system, at concentrations greater than about 0.5 mM, increased the effectiveness of pA(NeuAc) in inhibiting hemagglutination by 2- to 20-fold (enhancement is defined as $\Delta = (K_i^{HAI}{}_{[Neu2en]} = 0)/(K_i^{HAI}{}_{[Neu2en]})$. The enhancement of the polymer pA(NeuAc, $\chi^{NeuAc} = 1.0$) was greatest (ca. 20-fold) among tested polymers. b) The values for pA(NeuAc, $\chi^{NeuAc} = 1.0$) were measured as a function of the concentrations of three different Neu2en (Neu2en–NH_2, –OH, and –N_3). The concentrations of Neu2en required for half-maximal enhancement, $[Neu2en]_{1/2\Delta}$, were 70 μM, 300 μM, and ≥2 mM for Neu2en–NH_2, Neu2en–OH, and Neu2en–N_3, respectively. Although the data are limited, there seems to be a correlation between $[Neu2en]_{1/2\Delta}$ and values of K_i^{HAI}.

collection of experiments that both high affinity and a high degree of conformational flexibility are desirable properties of these polyvalent inhibitors. Steric stabilization may be a new strategy in the design of effective pharmaceutical agents. In the case of infectious agents, for example, we may, in principle, build polyvalent inhibitors of attachment that involve any molecule that binds tightly to the surface of the infectious agent. We are, remarkably, not limited to the

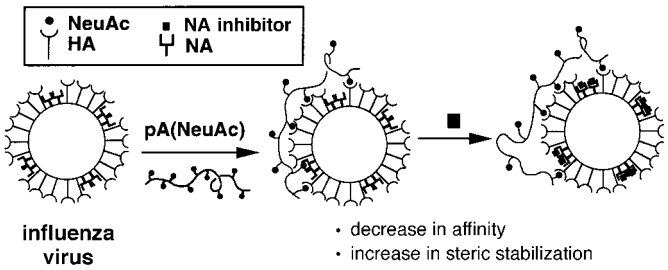


Figure 31. The hypothetical mechanism describing the modes of action of pA(NeuAc) in inhibition of influenza-mediated hemagglutination, and the effect of NA inhibitors on HAI activities of the pA(NeuAc). The presence of NA inhibitor is believed to release NeuAc from active sites of NA and/or HA sites, which may subsequently lead to expansion of gel layers.

polymers that present molecules that are directly involved in attachment. As an example, monomeric inhibitors of the surface enzyme neuraminidase, which is not involved in the attachment of influenza to cell, could nevertheless be presented on polymers to prevent attachment sterically.[362, 363] These polymers may function by binding to and establishing a hydrated gel layer on the surface of the virus or bacterium. Thus, polymers of a drug may change the original mechanism of action for that drug. Although there are currently no examples of such drugs, we have recently obtained some promising results using polymers of NA inhibitors that do not bind HA, but nevertheless inhibit hemagglutination effectively.[363]

We have described experiments with influenza virus whose results imply that steric stabilization may be an important mechanistic component of the anti-adhesive properties of large polymeric constructs targeting influenza virus. Given what we feel are the most likely of the possible biochemical mechanisms for steric stabilization, however, we believe that is it less likely that smaller constructs (such as Lee's glycoclusters[205]) exhibit significant steric stabilization of their targets. These constructs are comparable in size to antibodies (actually smaller than them), and the importance of steric inhibition in both remains to be established.

5.3. Polyvalent Promotion

In Section 5.2 synthetic agents that *inhibited* an undesired adhesion of two surfaces interacting polyvalently was described. There are few examples to date, however, of synthetic polyvalent molecules that trigger a signal upon binding, and thereby *promote* a desired interaction. Four examples are as follows:

1) Gong et al. described a polymer presenting multiple copies of GlcNAc that upon interaction with the surface of sperm triggered the acrosomal reaction (see Secion 4.4.1).[253]
2) Carrithers et al. described bivalent peptides targeted to G-protein-coupled receptors.[343] The ligand domain was either alpha-melanocyte stimulating hormone, an alpha-MSH receptor antagonist (alpha-MSH-ANT), or bombesin. These ligands were characterized in a functional melanocyte dispersion assay.
3) As described in Section 4.4.1, Dembo et al. described a series of bivalent antigen molecules that interacted with IgE molecules on the surface of a mast cell and thereby triggered degranulation.[226, 227, 324]

4) Multiple insulin molecules were attached to poly(acrylic acid) through an amide linkage.[364] This polyvalent insulin was more active (as a mitogen) on a per ligand basis than monovalent (natural) insulin.

5.4. Assaying the Polyvalent Interaction

There exist a number of methods for probing, assessing, and quantifying polyvalent interactions. Some assays may be direct measures of affinity; from these affinities, one may, in principle, extract free energies of interaction. Other assays may measure a complex aggregate of characteristics, only one of which is free energy of interaction: These other characteristics may include extent of hydration, ability to stabilize a molecule or surface sterically, and/or ability to cross-link multivalent receptors. We will not discuss here assays of polyvalent materials that go beyond examining their local effectiveness (i.e., assays that probe the often special pharmacodynamics and pharmacokinetics of large polyvalent materials relative to small monovalent ones, and even relative to large globular ones such as proteins).

5.4.1. Assays that Yield the Free Energy of Interaction of a Polyvalent System

To quantify a binding constant thermodynamically (i.e., to obtain a binding constant), the relative proportions of uncomplexed and complexed ligand or receptor must be directly or indirectly measured. Depending on the stability of the complex (related to its lifetime), different techniques can be used. Table 10 gives examples of a variety of techniques that have been used to measure polyvalent interactions.

Table 10. Techniques available to quantitate polyvalent interactions.

Technique	Example of an application	Enables measurement of			Comments
		affinity	inhibition[a]	kinetics	
hemagglutination inhibition assay[2]	1. inhibitors of the influenza–erythrocyte interactions, 2. antibody interactions with the surface of wide range of bacteria	no	yes	no	widely used; easily performed; in general, limited to inhibition constants greater than 1 nM
ELISA assay[278]	inhibitors of the influenza–erythrocyte interaction	yes	no	no	requires synthetic labeling of the polyvalent species; in general limited to dissociation constants greater than 10–50 nM
fluorescence-activated cell sorter[365]	antibody interactions with the surface of a cell	yes	yes, in principle	no	requires covalent modification of the antibody; based on the separation and subsequent quantification of bound and unbound forms
OPTCOL assay[335]	inhibitors of the influenza–erythrocyte interaction	no	yes	no	enables measurement under physiologically relevant conditions (collision velocity of cells, relative orientation, and other factors are controlled by the user); measurement involves a single cell and a single microsphere coated with viral particles, and the lower limit of measurable inhibition constants is less than 10^{-18} M.
affinity capillary electrophoresis[353]	dimers of vancomycin interacting with dimers of D-Ala-D-Ala	yes	no	no	this technique has much promise, and many extensions to other systems are possible and underway
surface plasmon resonance	antibody binding to synthetic surfaces presenting different densities of antigen (DNP, anti-DNP system);[366] influenza binding to immobilized hemagglutinin[367]	yes	no	yes	requires milligrams of the polyvalent material (other techniques in this table require 100 less in general); other related techniques include acoustic plate mode and surface acoustic wave, and are all based on the detection of small changes in dielectric constant near an interface
pipette suction	1. two erythrocytes bound by various agglutinins,[368] 2. CD2-containing jurkat cells adhering to LFA-3 in a planar membrane[369]	yes, in principle	yes, in principle	no	based on the quantification of the energy required to deform complementary cell surfaces during adhesion and separation; may be difficult to perform, or on the integrated forces required to separate two biological surfaces.
shear flow[370–372]	neutrophil interaction with surfaces derivatized noncovalently with different selectins	yes	yes, in principle	no	based on counting large number of events (stuck, non-stuck) under an optical microscope; probability of adhesion is measured as a function of flow rate of solution past surface
dissociation under influence of gravity[373, 374]	adhesion of vero green monkey kidney cells to growth surface	yes	no	no	time required to fall off surface is measured and correlated with strength of adhesion; centrifugal forces are almost always applied to hasten assay
optical microscopy counting aggregates[375]	sperm–egg interactions, and inhibition of these interactions	yes, in principle	yes	no	based on counting a large number of events (bound, unbound) following agitation; probability of adhesion can be measured as a function of concentration of inhibitor; difficult to perform
atomic force microscopy[376]	surfaces containing streptavidin interacting with surfaces containing biotin	yes	yes, in principle	no	highly sensitive (a single molecule–molecule interaction can be measured); expensive equipment required; covalent derivatization of small objects required
light scattering[377]	none to our knowledge	yes	yes, in principle	no	

[a] Inhibition of surface–surface interactions. The effectiveness of the assay is a result of both steric stabilization and enhanced affinity.

5.4.2. More Complex Assays of Polyvalency

In Vitro Aggregation

Some assays are available that can be used to measure the ability of a polyvalent ligand to aggregate a polyvalent receptor (precipitation, gel formation, aggregation). For example, a bivalent antibody can precipitate polyvalent proteins in immunoprecipitation assays (Figure 32). Although

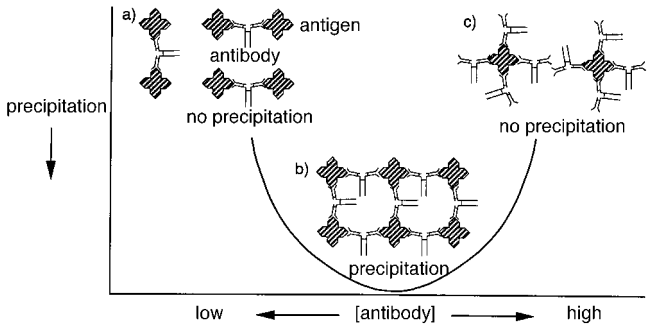


Figure 32. Influence of concentration of antigen in an immunoprecipitation assay. a) Formation of soluble antigen–antibody complexes in the presence of excess antigens. b) Formation of insoluble complexes (extensive lattice) near the equivalence point. c) Formation of soluble complexes in the presence of excess antibodies.

the affinity of the bivalent entity is important in determining the ability of the bivalent antibody to precipitate, other characteristics can be as, or even more, important: At low concentrations, the antibody fails to bind to the polyreceptor; at a certain concentration, precipitation occurs; at higher concentrations, each polyreceptor is surrounded by the antibody and precipitation again does not occur. In this example, affinity alone does not determine the pattern of precipitation.

In Vitro Inhibition of Aggregation

An example of an assay that measures the ability of a molecule to prevent two surfaces from interacting is the hemagglutination inhibition (HAI) assay (Figure 27). If the mechanism of inhibiting interaction was strictly based on competitive inhibition of all ligand–receptor pairs, the assay would only depend on the average affinity of each ligand–receptor pair in the polyvalent molecule (K_{avg}^{poly}). In such a case, an identical monovalent ligand (L) at a concentration $[L]^{mono} = K_{avg}^{poly}/K^{mono}$ should be equally effective in preventing two surfaces from interacting. As we have shown, the ability of a polyvalent inhibitor to prevent two biological surfaces from interacting may, however, involve steric stabilization, in which case molecular characteristics such as size, degree of hydration, and conformational flexibility play a role. Therefore, in cases where steric stabilization plays an important mechanistic role in inhibition, the concentration of the monovalent analogue of the ligand must be higher than the effective concentration of ligand in the polyvalent molecule ($[L]^{mono} > K_{avg}^{poly}/K^{mono}$) to be equally effective.

A limitation of the HAI assay stems from the minimum concentration of virus required to agglutinate erythrocytes.[2] This minimum concentration corresponds to approximately 1 nM of hemagglutinin protein in the assay vessel. The maximum value of K_i^{HAI} that can then be estimated reliably is therefore approximately 1 nM. We have recently described an assay in which two mesoscale particles (particles whose dimensions are approximately 0.1–100 μm) undergo a collision under the precise control of two independently controlled optical tweezers (optically controlled collision, OPTCOL; Figures 33–35).[335] This assay enables precise examination of the probability of adhesion under biologically relevant conditions. The biochemical and biomechanical

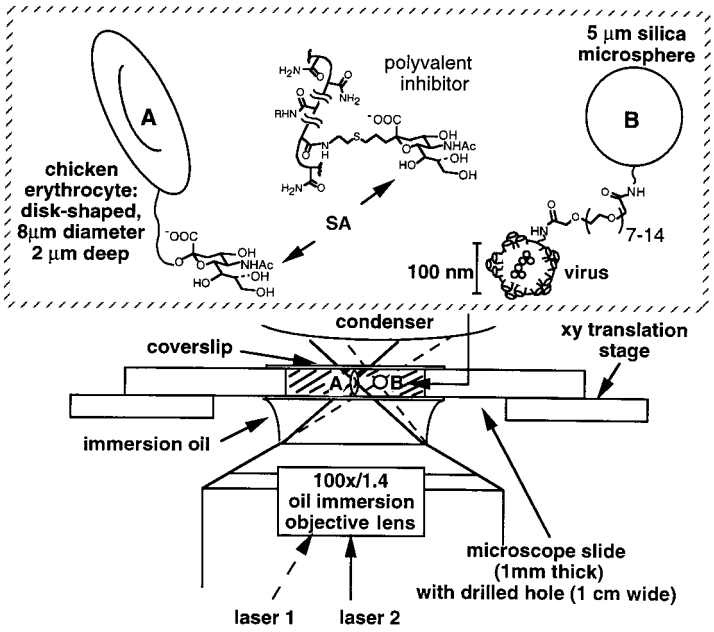


Figure 33. Schematic representation of the setup for the OPTCOL experiment, which uses lasers to quantitate the interaction between an erythrocyte ("A") and a microsphere covalently coated with influenza virus ("B"). A 1-cm hole is drilled in a microscope slide, and a "floor" is created with a coverslip. Particles A and B along with an inhibitor of the A–B interaction are added in buffer to this 100-μL "vessel". A second coverslip is placed to close the vessel, and the slide is placed under an inverted microscope. Two lasers are fed into the microscope. Each laser is focused through a lens. Particles A and B are "trapped" optically at the foci of the two lasers, and can be moved precisely and rapidly in three dimensions by manual or computerized translation of the foci. Shown are schematic drawings of the erythrocyte "A" (with SA covalently bound to various proteins and lipids embedded in the surface of the cell), the microsphere "B" (with virus tethered covalently to its surface), and the polymeric inhibitor (see also Figure 34).

determinants of adhesion between two biological objects following a collision are complex, and may vary from one system to another. The components of the solution, the relative orientation, the impact parameter, and the relative collision velocity are all under the user's control. These are precisely the variables that influence the probability of adhesion in vivo. We have illustrated the utility of the OPTCOL assay by evaluating the probability of adhesion of a single erythrocyte to a single virus-coated microsphere, both in the absence and presence of an inhibitor bearing sialic acid (Figure 34). The OPTCOL assay requires, in principle, only a single virus-coated microsphere and a single erythrocyte, and therefore the theoretical limit for association constants is as high as $10^{15}\,M^{-1}$. We assayed inhibitors using OPTCOL whose

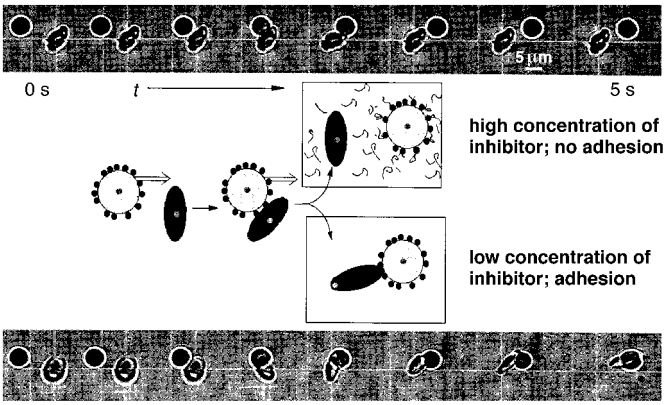


Figure 34. Schematic representation of the OPTCOL assay[328]. The virus-coated microsphere (in the mobile trap) is translated using one point of laser trap at a constant velocity and collides with the erythrocyte (in the stationary trap) above its center of mass; during this collision, the erythrocyte rotates. The erythrocyte remains in the stationary trap (the center of the right-hand crosshair) as the microsphere collides with it. The microsphere continues to translate linearly at constant velocity until it reaches a point approximately 20 μm from the original position of the erythrocyte. If the concentration of inhibitor is sufficiently high, the cell loses its initial contact with the microsphere and remains in the stationary trap as the microsphere translates past it. When no inhibitor, or too little inhibitor, is present, the cell adheres tightly to the passing microsphere, moves out of the stationary trap, and is "captured." Examination of the probability of adhesion as a function of concentrations of the inhibitor's sialic acid groups yielded an inhibition constant. We performed multiple repetitions of collisions for multiple pairs of microsphere and erythrocyte. Micrographs of the virus-coated microsphere before and after collision with the erythrocyte are shown.

values of $1/K_{avg}^{poly}$ varied from 1 nM to 10 mM. Four inhibitors all gave $K_{avg}^{poly} = 1$ nM, and were indistinguishable using HAI. We distinguished these four inhibitors easily using OPTCOL, and the best inhibitor prevented attachment 50% of the time at a sialic acid concentration of 35 pmol L^{-1}; it is the most potent known inhibitor of attachment of influenza virus to erythrocytes. We emphasize that this assay is empirical: It works because it can be calibrated by data measured in other ways.

OPTCOL is a versatile new bioassay for studying dynamic interactions in biochemistry. It offers an approach to investigating interactions between moving biological objects that is both quantitative and interpretable. The simplicity of the OPTCOL technique suggests broad applicability to the study of adhesion of mesoscale objects using relevant parameters in the areas of cell biology, microbiology, medicinal chemistry, and biophysics.

In Vivo Assays

In vivo assays measure the overall success of the inhibitor in protecting animals against infection; these assays include, but are not limited to, a measure of the inhibitor to prevent polyvalent interaction. The molecule may, for example, not only slow the rate of infection by blocking attachment to host receptors, but may slow the rate of clearance by blocking the clearance mechanisms. In the latter case, the polyvalent inhibitor might even increase overall infection! Overall success depends on these and other complex criteria such as toxicity, and often require that whole organisms be used for assays.

5.5. Future Prospects for Polyvalent Inhibitors

Polyvalent ligands directed at many of the naturally occurring polyvalent targets will undoubtedly be designed and synthesized. The mechanisms that are dominant for the success of monovalent agents are still important for poly-

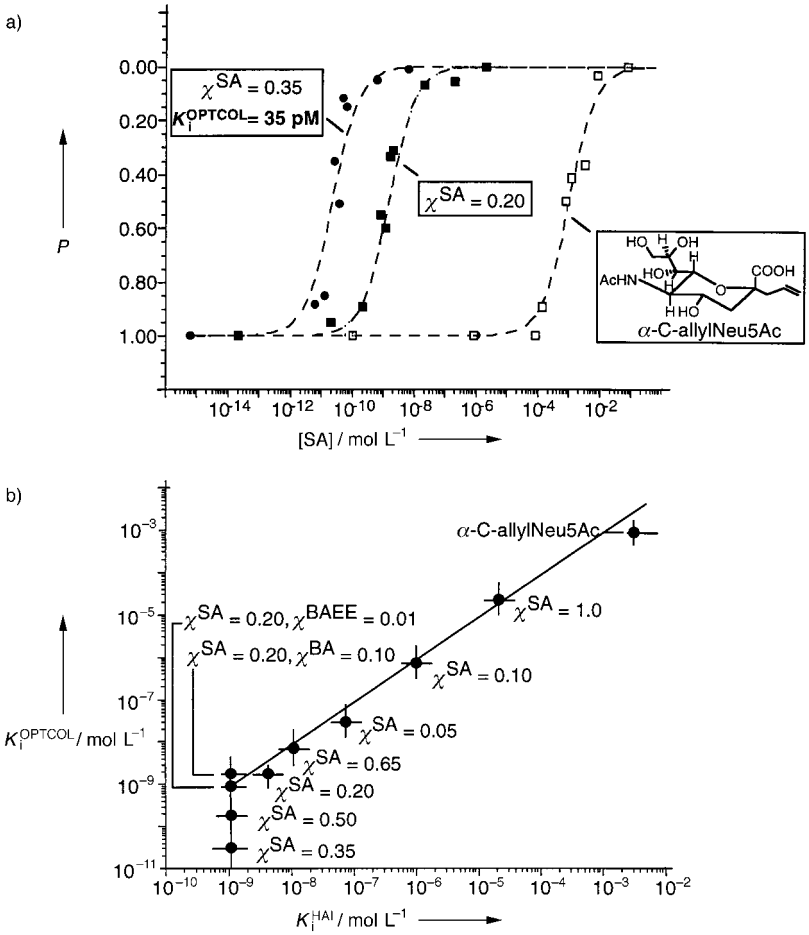


Figure 35. a) Typical inhibition curves obtained from OPTCOL experiments showing the probability of adhesion P as a function of the concentration of inhibitor (expressed as the concentration of SA groups).[328] The curves are fits of a binding isotherm (no cooperativity terms) with K_i as an adjustable parameter. We conservatively estimate the uncertainty in our measurements by fitting the data with two limiting values of K_i that exclude most (90%) of the data points. Probabilities were determined from at least 20 independent trials using 4–6 pairs of microsphere and erythrocyte. The values of K_i^{OPTCOL} were defined to be the value of [SA] for 50% probability of adhesion. b) The plot of measured values of K_i^{OPTCOL} against K_i^{HAI} show good agreement for $K_i^{OPTCOL} > 1$ nmol L^{-1}. BA = benzylamine, BAEE = bis(aminoethyl) ether. The error bars corresponding to a factor of ±2; the values of K_i^{HAI} were obtained from the literature.[5] For all inhibitors with $K_i^{OPTCOL} < 1$ nmol L^{-1}, K_i^{HAI} is approximately 1 nmol L^{-1}. For the solid line, $K_i^{HAI} = K_i^{OPTCOL}$ is valid.

valent ones, but at least one other mechanism not relevant to monovalent agents may also play a role: steric stabilization. Many issues must be clarified before polyvalency can be used for the design of and the prediction of outcomes of polyvalent inhibitors. Central questions include the following:

1) What is the best scaffold on which to present multiple copies of a ligand? The possibilities include both covalent frameworks (linear and branched polymers, dendrimers, proteins, and biodegradable beads) and noncovalent frameworks (liposomes). The "best" choice may be a function of the application. The conformational flexibility of the scaffold plays an important role, for example, in the ability of the polyvalent species to stabilize its target sterically, and in the value of B.
2) What are the chemical determinants of affinity for its target? What are the chemical determinants of the ability of the polyvalent species to stabilize its target sterically? These two questions relate to the best ways to design the spacing, rigidity, and hydrophilicity of the macromolecule. Despite extensive work, the precise rules that govern the specific (ligand–receptor) attachment of a polymer to a surface are not yet clear. The physical structure and arrangement of the group of "attached" polymers on the receptor-laden surface is also not clear from either a theoretical perspective, or an experimental one.
3) What are the chemical determinants of the toxicity of polyvalent inhibitors? These determinants may not be related in a simple way to those known to influence the toxicity of small molecules. The reason may be that small molecules are often designed, for a number of reasons, to be relatively hydrophobic, for example, to increase lifetimes in circulation by associating nonspecifically to proteins and thereby avoiding rapid filtration by the kidney. These small molecules are therefore often cleared predominantly by the liver. Polyvalent ligands can be hydrophilic and remain in circulation for prolonged periods because of their size. By varying the sizes of these polyvalent species, perhaps by varying the biodegradability of the linkages connecting its different segments, these molecules can be designed to be cleared by the liver, spleen, or kidney. The chemical determinants of toxicity will depend, in part, on the route of clearance.
4) What are the best ways to deliver polyvalent ligands? Because these polyvalent species are often large, they may not pass passively and rapidly through hydrophobic membranes; injection may be more appropriate than ingestion or transdermal delivery for applications within the vascular space, for instance. These issues of delivery are related to those for protein-based drugs. We are just beginning to understand some of these issues, while others remain completely unexplored. The kinetics and biophysical mechanisms of passage of polymeric molecules across porous membranes is also relevant here, and remains an area of exploration in biophysics and statistical mechanics. The same challenges that are faced in the delivery of polyvalent materials, however, serve as advantages from a pharmacokinetic perspective. That is, once a polyvalent molecule of sufficient size is delivered to the vascular compartment, the biological lifetime is expected to be greater than that of the monovalent analogue. Lengthening lifetimes has several advantages including reduced dosing frequency (and therefore increased compliance) and the ability to use materials that have intrinsically lower therapeutic indices (with the same dosing frequency, the difference between peak and trough concentrations of the active agent will be less than that for agents cleared more quickly).

Applications of polyvalent molecules as pharmaceuticals should be possible, and should open new doors in the industry. Although this review is not the forum for exhaustively presenting new areas of application, describing two such areas may be illustrative of the potential of polyvalency.

Any biological surface that interacts with another biological surface through multiple attachment points can, in principle, be a target for polyvalent drug design. That is, naturally polyvalent interactions can be antagonized with synthetic polyvalent molecules. One example may be that molecules that are composed of repeating units of small moieties essential for viral attachment and entry may provide a general strategy for the inhibition of viral infection. Since steric stabilization is expected to play a role in the mechanism, inhibitors presenting multiple copies of a single ligand may be active even against virus particles that may use more than one, or alternative, receptors for primary viral attachment (e.g. HIV). A second example may be that the interactions between platelets, or between denuded endothelia and platelets, may be inhibited using a polyvalent inhibitor of integrin binding. These agents might be particularly useful if more than one type of ligand is incorporated into the backbone such that only regions expressing more than one type of receptor (i.e., sites of pathology) are targets of this highly specific polyvalent agent. Such agents may find uses in the treatment of pathological clotting (e.g. deep-vein thrombosis, arterial thrombosis in a coronary artery leading to myocardial infarction, formation of a clot in a fibrillating atrium leading to myocardial infarction or stroke).

A second type of application is in agonizing interactions that are not naturally polyvalent with synthetic polyvalent molecules. For example, one may design a molecule that presents multiple copies of acetyl choline or epinephrine that binds very tightly to cholinergic and adrenergic receptors, respectively. These polyvalent materials may be useful agonists of the parasympathetic and sympathetic nervous system. Since the affinity of these agents will most likely be a function of the density and accessibility of receptors on a surface, there is a likelihood of designing molecules that are targeted to cells not according to whether or not they express these particular classes of receptors, but rather according to the concentration at which they are expressed. This strategy may then allow specificity to be built into a pharmaceutical agent that is physically independent of the details of the individual ligand–receptor interaction.

6. Conclusions

Polyvalency occurs broadly in biological systems, but remains largely unappreciated by those who study either biology or design and synthesize chemical agents intended to

interact with biological processes. Polyvalent interactions in biology suggest new targets for interaction using pharmaceuticals that are themselves polyvalent. The greatest barriers to the design of effective polyvalent pharmaceuticals may be a deficiency in the tools to assay polyvalent interactions, and an incomplete understanding of the theoretical framework underlying it. Successful design of synthetic polyvalent systems requires a detailed understanding of the delicate balance between entropy and enthalpy. Chemists and biochemists understand enthalpy well; they do not understand entropy, especially conformational entropy, as well.

Polyvalency confers characteristics on a system that are not present in monovalent interactions. For example, the following are possible with polyvalency: achieving very tight binding from ligands with modest or low surface area; grading (graduating) biological responses, possibly with nonlinear dependence on the strength of the signal; creating new interactions by mixing, matching, and multiplying existing ones; achieving large conformal contact between biological surfaces, such as those on cells, and enabling efficient communication; inducing changes in the morphologies of large structures such as cells; signaling by the induction of large conformational changes of molecules; inducing changes in the distribution of molecules in a membrane; signaling through oligomerization or dimerization; and inhibiting undesired interactions, such as those between invading pathogens (which are themselves polyvalent) and their targets.

The study of polyvalency may provide new targets and new strategies for the design of pharmaceutical agents. Although the detailed examination of the active site of receptors as an aid in the design and synthesis of tightly binding monomers will continue, new strategies will have to be developed to design scaffolds that hold multiple designed ligands together. The degree of flexibility, the extent of hydration, and the size of the scaffold will play important roles in the design of successful polyvalent pharmaceuticals. In fitting with the strategy most commonly used with small pharmaceuticals, the first polyvalent ligands will probably be designed to be inhibitors of some undesirable process. The first areas of application will probably be in the design of inhibitors of attachment of both viruses and bacteria. Other applications, such as those in cancer (e.g. inhibition of metastasis), are more speculative. Eventually, as with the current wave of protein-based drugs, polyvalent ligands may be designed that promote desired effects, such as modulating the immune system to promote the clonal expansion of specific cellular components.

Assaying a polyvalent interaction remains a fundamental challenge. Functional assays may be more important for polyvalent interactions than for monovalent ones. That is, assays that measure only the affinity of a polyvalent molecule for its target may not be optimally useful in predicting the biological effect. Useful assays should weigh the characteristics of the polyvalent ligand that are important for achieving the desired goal. For example, an effective bivalent molecule for the purpose of aggregating a polyvalent receptor (such as for purification of cells on a large scale by immunoprecipitation, where antibodies are prohibitively expensive) might be composed of two ligands linked through a long flexible linker. An effective bivalent molecule for the purpose of bivalent binding to a single polyvalent receptor (for example, for tight binding to the surface of a pathogen) might be two ligands spaced by a rigid linker of a length that approximates the spacing of receptor sites on the surface of the polyvalent receptor. In this example, both purposes involve the design of a bivalent ligand; the principles of design depend on the purpose. The assay, therefore, must reflect the characteristics important in the use of the polyvalent species.

The inhibition of adhesion of influenza virus A (X-31) to erythrocytes by polyacrylamide presenting multiple sialic acid groups is currently the most extensively studied system of polyvalent inhibition. The most important conclusion that we reach through this set of studies is that the polyvalent, polymeric inhibitors function through at least two distinct, limiting mechanisms: high-affinity, entropically enhanced binding and steric stabilization (defined as the "blocking" of a surface through the development of a large, hydrated "gel" layer). Our most effective inhibitors in studies of the model system involving influenza are effective at inhibiting the interaction between influenza virus and erythrocytes at approximately $10^7 - 10^8$ times lower concentration than the corresponding monomer. In one detailed study in this series of polymers, approximately 10^6 of the total enhancement was due to increased affinity of the polymer for the surface of the virus, and 10^2 was due to steric stabilization.[378]

Appendix: Abbreviations Used in the Text

α	cooperativity constant, defined by Equations (7) and (8b)
β	cooperativity constant appropriate for polyvalent interactions, defined by Equations (9) – (11)
CD4	protein found on the surface of helper T-cells
CD8	protein found on the surface of cytotoxic T-cells
CRBP	cellular retinol binding protein
DNP	dinitrophenol
ELISA	enzyme-linked immunosorbant assay
Fab	monovalent, antigen-binding arm of an antibody
Fc	portion of antibody that is structurally different from the Fab and relatively constant in sequence within an antibody class; the "tail" of an antibody
Gal	galactose
GalTase	galactosyltransferase
GlcNAc	*N*-acetylglucosamine
HA	hemagglutinin
HAI	hemagglutination inhibition assay
hGH	human growth hormone
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IgE	immunoglobulin class E
IgG	immunoglobulin class G
IgM	immunoglobulin class M
MHC	major histocompatibility complex
MSH	melanocyte stimulating hormone
NA	neuraminidase
Neu5Ac	5-acetylneuraminic acid
OPTCOL	optical collision assay
PDGF	platelet derived growth factor

RXR	retinoid X receptor
SA	sialic acid
sLe^X	sialyl $Lewis^X$
SH2	Src homology 2 protein
TCR	T-cell receptor
TNF	tumor necrosis factor
VSV	vesicular stomatitis virus

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