Chapter 14
Engineering Multivalent and Multispecific Protein Therapeutics

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14.1 Biomedical Applications of Multivalent and Multispecific Proteins

Proteins have attracted great interest as therapeutic agents due to their high-binding affinity and specificity to clinical targets of interest [1]. The last several decades have witnessed a surge in our ability to engineer protein drug candidates, from improved algorithms for rational design [2, 3] to new platforms for directed evolution [4–7]. In parallel, multivalent or multispecific proteins have shown great therapeutic potential compared with their monospecific protein counterparts due to increased efficacy or enhanced selectivity toward sites of disease [8–11]. Nature commonly exploits multivalency and multispecificity to regulate numerous physiological processes [12, 13]; applying these concepts to protein drug discovery offers exciting new avenues for clinical development. A thorough understanding of the thermodynamic and kinetic principles underlying multivalency and multispecificity is essential to develop therapeutic proteins that promote these improved biological effects. In addition, this understanding allows researchers to effectively affirm or challenge proposed molecular mechanisms for existing protein therapeutics. This chapter will present the underlying principles of multivalent or multispecific protein therapeutics as outlined below, where each concept will be accompanied with example case studies from literature:

14.2 Multivalency and Binding Avidity.
14.3 Tuning Selectivity through Multivalency and Multispecificity.
14.4 Biological Principles for Multivalent and Multispecific Protein Design.

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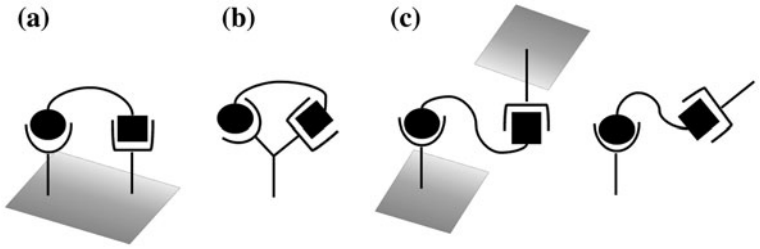


Fig. 14.1 Targeting strategies for multivalent and multispecific proteins. *Circles* and *squares* represent individual binding sites that can be either identical (monospecific) or different (bispecific). Ligand can simultaneously bind: **a** two receptors on the same cell surface; **b** two epitopes on the same receptor; and **c** two receptors on separate surfaces or in solution

14.2 Multivalency and Binding Avidity

Before considering the design and engineering of proteins for clinical applications, one must first have a clear understanding of the underlying concepts behind binding affinity and avidity effects stemming from *multivalency* and *multispecificity*. We refer to a molecule as multivalent if it contains multiple binding sites, and refer to it as multispecific if the sites bind to different targets, or different epitopes within a target. The reader should note that multivalency by definition encompasses multispecificity; that is, a molecule with more than one binding site will always be multivalent, but not necessarily multispecific. A multispecific protein is, however, always multivalent. Figure 14.1 shows examples of how the two binding sites of bivalent molecules can interact in different ways with their respective targets. For our discussion, a target is a biomolecule, such as a receptor, that has clinical relevance and whose biological activity mediates disease pathology.

In this section, we present the basic kinetic and thermodynamic models for ligand–receptor binding, beginning with a monovalent interaction and expand these models to include multivalency. More detailed analyses are offered in various textbooks and reviews as cited; this introduction should give the reader a sense of the physical factors contributing to the advantageous properties of multivalent proteins, and how information from these models can guide the design and engineering of protein therapeutics.

14.2.1 Equilibrium Binding Constant, K_D

A monovalent binding interaction between a therapeutic protein, which we call ligand (L), and the target molecule, which we call receptor (R), is described by the following equation:

$$L + R \underset{k_{off}}{\overset{k_{on}}{\longleftrightarrow}} LR \tag{14.1}$$

where LR represents the 1:1 complex of L and R, and k_{on} and k_{off} are the rate constants for association and dissociation, respectively. The association and dissociation rates are defined as follows:

$$r_{association} = k_{on}[L][R] \tag{14.2}$$

$$r_{dissociation} = k_{off}[LR] \tag{14.3}$$

At some time after the start of the reaction, the system reaches equilibrium concentrations of L, R, and LR, reflecting the relative values of k_{on}, k_{off}, and the initial concentrations of L and R: $\frac{d[L]}{dt} = \frac{d[R]}{dt} = \frac{d[LR]}{dt} = 0$. The concentrations of free L and R, and LR remain in a dynamic equilibrium, in that $r_{association} = r_{dissociation}$. We can therefore say that:

$$k_{on}[L]_{eq}[R]_{eq} = k_{off}[LR]_{eq} \tag{14.4}$$

which can be rearranged to:

$$\frac{k_{off}}{k_{on}} = \frac{[L]_{eq}[R]_{eq}}{[LR]_{eq}} \tag{14.5}$$

The subscript "eq" indicates that the concentrations are at equilibrium. The ratio of k_{off} over k_{on} describes the strength of the interaction and is defined as the equilibrium binding constant (K_D) of L and R.

$$K_D = \frac{k_{off}}{k_{on}} [=] \text{mole}/L \tag{14.6}$$

As shown in Eqs. 14.5 and 14.6, the K_D represents the ratio of the individual concentrations of L and R to the concentration of the LR complex at equilibrium. From this definition, a lower K_D corresponds to stronger binding affinity, while a higher K_D corresponds to weaker binding affinity. Measurements of K_D, k_{on}, and k_{off} provide common metrics for describing the binding affinity and kinetic binding parameters of a molecular interaction. These properties can play a direct role in the biological efficacy of a protein therapeutic [14–16]. Thus, there has been great interest in determining the relationships between binding affinity and biological function, and how alterations in these parameters can be leveraged in protein engineering to direct a biological outcome. Figure 14.2 illustrates the effect of K_D on the percent of R converted to LR at equilibrium, for a range of L concentrations. Typical K_D values range from μM for enzyme–substrate interactions, to nM and pM for cell surface receptor binding events, and even fM in rare cases such as biotin–streptavidin, underscoring how nature has optimized binding affinities for different biological contexts.

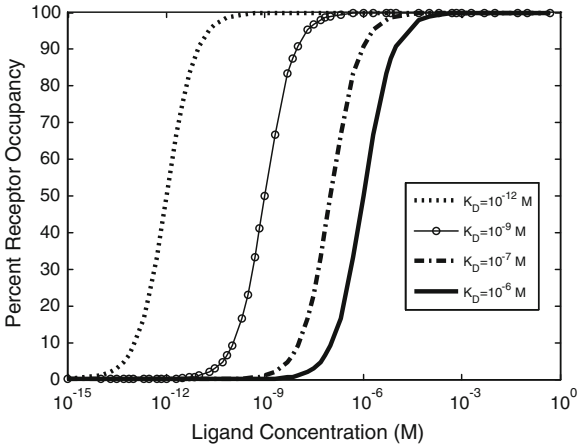


Fig. 14.2 Ligand–receptor binding for a range of K_D values

14.2.2 Thermodynamic Considerations in Binding

The laws of thermodynamics dictate protein biophysical properties, including folding, structure, and binding energetics. Our discussion on thermodynamics will be centered around the Gibbs free energy (ΔG) of binding, which can be thought of as the energy (kcal/mol) gained from or lost to the environment in a binding event. Studies of protein–protein interactions often explore how altering amino acid sequence and protein structure affects ΔG, consequently identifying important molecular contributions to binding affinity. The following equation relates the Gibbs free energy to the K_D:

$$\Delta G = RT \ln(K_D) \tag{14.7}$$

where R is the ideal gas constant, with units of kcal/(mol * K). The same equation can be rearranged to obtain:

$$K_D = e^{\frac{\Delta G}{RT}} \tag{14.8}$$

The implication of Eqs. 14.7 and 14.8 is that the binding affinity is dependent on the energetics of binding as well as temperature. Here, a negative ΔG indicates an energetically favorable binding reaction that will spontaneously proceed in the forward direction, and a positive ΔG indicates an unfavorable one. The simplest way to understand the relationship between energetics and kinetics is through a reaction coordinate diagram, as shown in Fig. 14.3.

On the reaction coordinate, the relative energies of free L and R and the complex LR are measured as ΔG. The LR complex state must be lower in energy than the free states in order for the binding interaction to be favorable. This corresponds to a negative sign for ΔG if defined as $G_{\text{complex}} - G_{\text{free}}$. The curve represents the reaction pathway and is indicative of the equilibrium amounts of L, R, and LR as defined by the K_D value. In this case, the binding event is comprised of two steps. The first step (1), which requires some starting energy $E_{a,\text{assoc}}$,

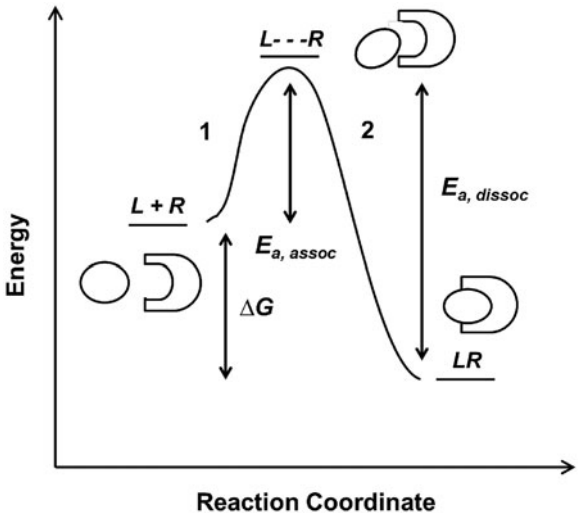


Fig. 14.3 Reaction coordinate diagram of a monovalent binding interaction

arranges the ligand and receptor an optimal position and conformation for binding. The starting energy $E_{a,\text{assoc}}$ arises from thermal fluctuation of the molecules in solution. In the second step (2), the *LR* complex formation releases an energy that drives the binding interaction forward. The same principles drive the dissociation reaction (with an $E_{a,\text{dissoc}} = E_{a,\text{assoc}} + \Delta G$), until the system reaches equilibrium between the two states. The relative amount of energy $E_{a,\text{assoc}}$ or $E_{a,\text{dissoc}}$ required for association or dissociation, respectively, determines the probability of the molecules to successfully move forward or backward on the coordinate diagram. This probability is reflected in the rate constants, and hence K_D. In reality, protein–protein interactions are often more complicated than the simple curve and two-step reaction illustrated above; for example, a reaction mechanism may contain multiple intermediate steps or require cofactors to facilitate ligand–receptor binding. However, the reaction coordinate provides good intuitive understanding of binding energetics and kinetics.

We can now further describe ΔG of binding in more physical terms, namely ΔH (enthalpy), temperature, and ΔS (entropy) of binding. Note that both ΔH and ΔS are state functions, meaning that regardless of the simplicity or complexity of the reaction mechanism, their values depend only on the relative energies between the free (initial) and complexed (final) states.

$$\Delta G = \Delta H - T\Delta S \tag{14.9}$$

Formally, enthalpy is defined as the sum of internal energy of a system (energy required to create the system) and the work the system exerts on its surroundings. In the context of protein–protein interactions, we can think of enthalpy as a measure of the electrostatic and geometric complementarity between two proteins. Enthalpic contributions consist of molecular interactions of both binding partners that facilitate stronger noncovalent binding, as well as molecular interactions between the

proteins and the surrounding environment. A negative ΔH value indicates an exothermic reaction (binding releases energy) while a positive ΔH value indicates an endothermic reaction (binding requires energy). Since a favorable binding event necessitates a negative ΔG value, a larger negative ΔH value correlates with better complementarity between the two binding partners.

Entropic contributions describe changes in the degrees of freedom of movement of L, R, and their surrounding solvent upon binding. In order to form an LR complex, L and R must adopt optimal conformations relative to each other, reducing the entropy of both proteins ($S_{\text{final}} - S_{\text{initial}} < 0$); from Eq. 14.9, we see that this results in a negative ΔS value, leading to a more positive, and therefore less favorable ΔG value. To better explain entropy, we divide ΔS into more specific subtypes [17–20]:

$$\Delta S = \Delta S_{\text{translation}} + \Delta S_{\text{rotation}} + \Delta S_{\text{conformation}} + \Delta S_{\text{solvation}} \tag{14.10}$$

Translational and rotational entropy describes the protein's ability to move in three-dimensional space. In nature, the reduction of translational degrees of freedom is necessary for facilitating many biological processes; a canonical example is the movement of proteins along DNA [21]. In our monovalent binding example, LR complex formation restricts the movement of ligand L to a particular volume occupied by the receptor R, and vice versa. Conformational entropy describes the protein's thermal fluctuations around its native state. This idea is based on the prevalent view that free ligand and receptor are able to sample a range of orientations around their primary native states, which are optimal for binding to each other. In the context of Fig. 14.3, one could imagine many slightly different free protein conformations at various energies, with an average energy as indicated by the horizontal line at $L + R$. Upon binding, the primary conformations of both ligand and receptor are stabilized, greatly reducing their accessibilities to the initial range of conformations. Solvation entropy refers to the energy required to arrange water molecules around each other and around a protein. Upon protein binding, formation of the binding interface reduces the surface area between protein and water. Given that water molecules experience the greatest entropy when they are allowed to arrange around each other, protein binding results in a positive change in solvation entropy.

Using Eq. 14.9, we can rewrite Eq. 14.8 as:

$$K_D = e^{\left(\frac{\Delta H}{RT} - \frac{\Delta S}{R}\right)} \tag{14.11}$$

This representation shows that manipulations of enthalpy and entropy alter the free energy, and hence the binding affinity, of a given protein toward its intended target molecule [22, 23]. To enhance enthalpic contributions one might, for example, mutate amino acid residues within a binding interface to increase the charge or size complementarity. However, these effects are difficult to predict *a priori* using rational design; changes in the enthalpy of a protein–protein interaction often have unintended effects on entropy, and vice versa, all of which influence the K_D value [19, 24].

14.2.3 Binding Avidity

We now extend the kinetic model to the case of a bivalent protein ligand; models describing proteins of higher order valency will follow the same principles outlined below. Bivalency increases the complexity of ligand–receptor binding kinetics through the physical tethering of one binding site in close proximity to another. The ligand is now a molecule containing two binding sites, each binding to one receptor molecule on a cell surface. Hence, the binding equation is altered as follows:

$$L + R \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\longleftrightarrow}} LR \text{ for monovalent} \tag{14.12}$$

$$L_2 + R_2 \underset{k_{\text{off,bi}}}{\overset{k_{\text{on,bi}}}{\longleftrightarrow}} L_2R_2 \text{ for bivalent} \tag{14.13}$$

where $k_{\text{on,bi}}$ and $k_{\text{off,bi}}$ are the *apparent* rate constants of the bivalent ligand to the receptor. The term apparent is used to indicate empirically determined parameters that cannot distinguish between the individual receptor binding events of each ligand component. Often, there is an increase in the apparent affinity of a bivalent protein compared with the affinities of its monovalent components, a phenomenon known as *avidity*. To illustrate this point, we introduce the sequential binding reactions shown in Fig. 14.4, a concept originally proposed by Jencks [25]. In this schematic, ligands *A*, *B*, and *A*—*B* are free-floating, while their receptors are constrained to a surface (i.e., cell membrane) at a certain density. *A* and *B* can be identical (monospecific ligand) or different (bispecific ligand); the analysis is the same regardless of their identities. First consider Fig. 14.4a, where the binding sites *A* and *B* are untethered. In this case, the receptor binding events are independent monovalent interactions that can be described by Eq. 14.12. Hence, the interactions of *A* and *B* with their receptors are defined by their affinities $K_{D,A}$ and $K_{D,B}$, respectively.

Now, we consider the more complex scenario in Fig. 14.4b, where *A* and *B* are tethered. The binding of the first site, whether it be *A* or *B*, to its target receptor follows the same monovalent binding kinetics as in Fig. 14.4a (State 1) and assumes that the binding events are independent. Below we present the binding equations of State 1 going to State 2:

$$A - B + R_A - R_B \underset{k_{\text{off},A}}{\overset{k_{\text{on},A}}{\longleftrightarrow}} R_B - R_AA - B \,(\text{State 1} \rightarrow \text{State 2a}) \tag{14.14a}$$

$$A - B + R_A - R_B \underset{k_{\text{off},B}}{\overset{k_{\text{on},B}}{\longleftrightarrow}} R_A - R_BB - A \,(\text{State 1} \rightarrow \text{State 2b}) \tag{14.14b}$$

In Eqs. 14.14a and 14.14b, the receptors are represented as $R_A - R_B$ to indicate that they are on the same cell surface. In State 2, one ligand binding site is bound to a receptor, and thus, the cell surface, while the other is brought to the surface but still unbound. The second binding site is thus constrained to a volume defined by

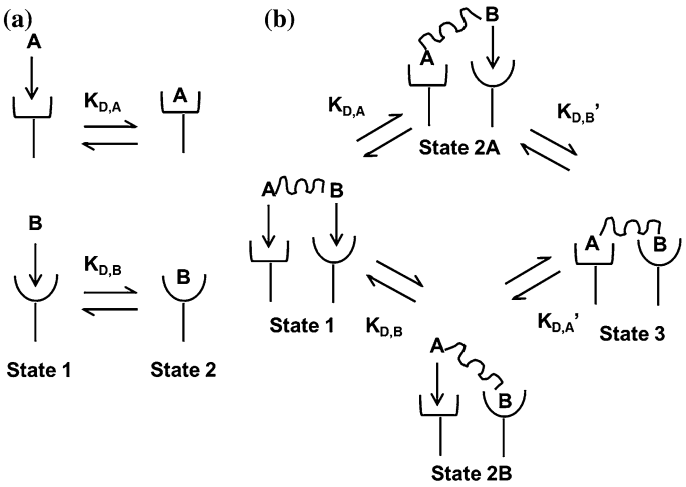


Fig. 14.4 Binding schematic of ligands *A* and *B* with their respective receptors. **a** Untethered *A* and *B* bind receptors independent of each other with affinities $K_{D,A}$ and $K_{D,B}$. **b** Tethered *A*—*B* binds receptors in multiple states. State 1: both binding sites are unattached. State 2a,b: either *A* or *B* binds independent of the other with affinities $K_{D,A}$ and $K_{D,B}$. State 3: second binding event depends on the first. Either *A* or *B* binds with affinities $K_{D,A}{}'$ and $K_{D,B}{}'$. Figure adapted from Jencks [25]

the space available to the linker joining *A* and *B* (State 2a and 2b). Although only one of the binding sites is bound to a receptor, the second binding site is more favorably placed near its receptor, increasing its probability of undergoing a binding interaction. In effect, we have transitioned from an intermolecular binding event to an intramolecular binding event [18, 26] as described by Eqs. 14.15a and 14.15b.

$$R_B - R_A A - B \underset{k_{\text{off},B'}}{\overset{k_{\text{on},B'}}{\longleftrightarrow}} R_A A - BR_B (\text{State 2a} \rightarrow \text{State 3}) \tag{14.15a}$$

$$R_A - R_B B - A \underset{k_{\text{off},A'}}{\overset{k_{\text{on},A'}}{\longleftrightarrow}} R_A A - BR_B (\text{State 2b} \rightarrow \text{State 3}) \tag{14.15b}$$

How do the apparent affinities $K_{D,A}{}'$ and $K_{D,B}{}'$ differ from the monovalent affinities $K_{D,A}$ and $K_{D,B}$? If we look at the binding events that occur from State 2 to State 3, we see that the effective concentration of the second binding site around free receptors has dramatically increased (Fig 14.5). It is important to recognize that this effective concentration can be orders of magnitude greater than the concentration of free ligand in solution, to the point where $K_{D,A}{}' \ll K_{D,A}$ and $K_{D,B}{}' \ll K_{D,B}$. In other words, at equilibrium, the dominant species in solution are those in 14.15a and 14.15b representing State 2 and State 3. This phenomenon explains the avidity effect: *when the effective concentration of the second ligand is so large that it drives the subsequent association reaction forward, the rate of*

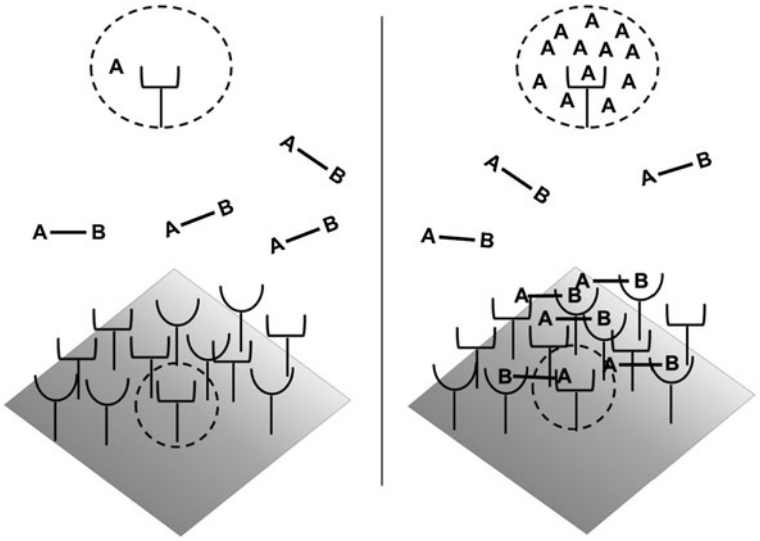


Fig. 14.5 Avidity effects due to increased effective concentration. *Left*: Before binding, the concentration of binding sites as seen by a cell surface receptor is equivalent to the ligand concentration in solution. *Right*: Upon monovalent binding of site *B*, the effective concentration of binding site *A* seen by the receptor is vastly increased, driving the association reaction forward

complete dissociation of the ligand from the cell surface is greatly reduced compared with that of monovalent binding interactions.

Now, we relate increased effective concentration to increased overall affinity due to bivalency. C_{eff} is a term describing the enhancement of effective concentration of either *A* or *B* due to their tethering [19, 26]. We define C_{eff} as the ratio of the ligand–receptor affinity of the monovalent binding event compared with their apparent affinity in a bivalent binding event:

$$C_{\text{eff}} = \frac{K_{D,A}}{K_{D,A'}} = \frac{K_{D,B}}{K_{D,B'}} \tag{14.16}$$

If $C_{\text{eff}} > 1$ M, the bivalent protein has an increased apparent affinity to the cell surface due to avidity effects. This sequential binding event can be represented by an overall affinity $K_{D,AB,\text{bi}}$.

$$K_{D,AB,\text{bi}} = K_{D,A} * K_{D,B'} = K_{D,B} * K_{D,A'} \tag{14.17}$$

Since the reaction going from State 2 to State 3 is unimolecular, $K_{D,A}{}'$ and $K_{D,B}{}'$ are unitless. $K_{D,AB,\text{bi}}$ therefore has units of M.

Avidity can increase the apparent affinity of a multivalent ligand to be orders of magnitude greater compared with that of its monovalent components. As such, multivalency is a common phenomenon exploited by nature to enhance the affinities of protein ligands to their binding partners, eliminating the need to

produce large amounts of monovalent protein or to evolve a single binding domain with high affinity. As examples, many growth factors utilize multivalency to achieve overall binding affinities in the nM–pM range. Vascular endothelial growth factor (VEGF) is a disulfide-linked homodimer that binds to two VEGF-receptor 2 (VEGFR2) molecules on the cell surface and induces their dimerization, stimulating downstream cell-signaling pathways [27, 28]. The strong affinity of VEGF for its receptor is in part due to its bivalent binding properties; when one VEGFR2-binding site was eliminated through directed mutagenesis, the affinity of VEGF was reduced by two orders of magnitude [28]. Hepatocyte growth factor (HGF) is a multidomain-soluble ligand that also binds to and dimerizes its cognate receptor (c-MET) with pM affinity [29]. Recent studies have shown that individual domains of HGF contribute weakly to c-MET binding, resulting in overall avidity effects when all domains are present [30, 31]. As a further demonstration of avidity effects in this system, a truncated HGF fragment termed NK1, despite its ability to dimerize the receptor, binds c-MET with a reduced affinity (nM) and is orders of magnitude less efficacious in inducing c-MET activation compared to HGF [30, 32]. In the context of protein therapeutics, the most well-studied natural multivalent protein is the antibody. Antibodies are modular, containing a constant (Fc) domain that mediates immune system activity, and two identical antigen-binding regions consisting of heavy and light variable chains. Natural antibodies are bivalent, but monospecific, in that these two antigen binding sites interact with the same target. Antibodies have been specifically evolved, either by nature or in the laboratory, for high-affinity recognition of a wide range of antigen targets and thus are applicable across a broad spectrum of medical problems [9, 10, 33].

14.2.4 Linkers and Avidity

Designing multivalent proteins with the desired kinetic properties can be carried out by: (1) engineering the binding sites themselves, (2) optimizing the linker that tethers the monovalent components, if applicable, or (3) both. Methods for altering protein–protein interactions have been extensively reviewed, and include both rational design and directed evolution [2–7]. In addition, much effort has gone into linker development, as optimal flexibilities and length requirements differ among systems [34, 35]. Linkers can strongly influence the avidity of multivalent protein interactions through entropic effects, and thus, their optimization is essential for achieving desired levels of therapeutic efficacy. In this subsection, we will describe the relationship between linker entropy and ligand-binding kinetics.

To introduce linker thermodynamics, we will use a polymer chain as an example of a model linker. Linkers can assume many different geometries and chemical compositions but are often linear peptide chains of varying length [36]. Entropy demands that the linker exist in random orientations in solution; this randomness is caused by thermal fluctuations. However, there is a deformation cost to bending that opposes entropy. This property, which depends on linker

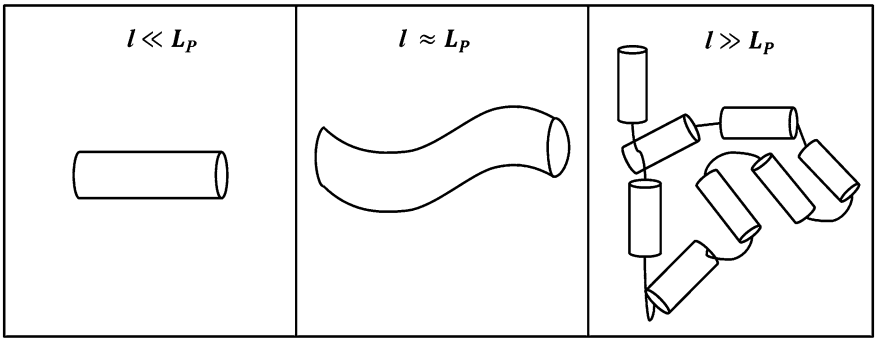


Fig. 14.6 Polymer chain at three different length scales. As l increases, deformation of the chain by thermal fluctuations randomizes the orientation

composition, is referred to as its elasticity. To illustrate this concept, we introduce a parameter called the persistence length L_P, whose informal definition is the length over which a polymer strand is relatively straight, and is a measure of the strand's elasticity or rigidity. Formally, it is the ratio of the linker's elastic energy over thermal energy. A small L_P indicates a flexible strand since the deformation cost is easily overcome by thermal fluctuations. Similarly, a large L_P indicates a rigid strand, since the deformation cost is large, and the strand is not easily bent to different conformations. In Fig. 14.6, we present three different views of a polymer strand: at length $l \ll L_P$, the strand looks perfectly straight; at $l \approx L_P$, the polymer is still relatively straight, but one sees the initial deformation of the strand; at $l \gg L_P$, the linker can now be seen as a collection of rigid segments of length L_P where the hinges allow each segment freedom of translation and rotation, and linker orientation can be approximated as a random walk in three dimensions.

To understand the mechanism of how linkers influence binding affinity, we will explore avidity effects in the context of thermodynamics. The binding of the first site in Fig. 14.4b (State 1) lowers the translational, rotational, and conformational freedom of both molecules in the complex, represented by ΔS (see Eq. 14.10). As a result, the effective concentrations of the other ligand-binding sites are increased; in other words, the translational and rotational space that the tethered sites can sample is restricted to a smaller volume around their receptors. Thus, the entropic penalty for constraining any tethered binding site and its receptor in proximity with each other is paid to an extent by the binding of the first ligand. The ΔG of binding is therefore decreased for any subsequent interaction, lowering the apparent K_D for a multivalent ligand compared with that of a monovalent ligand. The requirement for avidity is thus $\Delta G_{\text{multi},N} < \Delta G_{1,\text{mono}} + \Delta G_{2,\text{mono}} + \cdots + \Delta G_{N,\text{mono}}$ [18], where $\Delta G_{\text{multi},N}$ is the total energy loss upon binding of the multivalent ligand with valency N, and $\Delta G_{1,\text{mono}}$ is the total energy loss upon binding of an independent monovalent site 1. Linker length and elasticity determines the magnitude of entropy loss for each binding event between a multivalent ligand and

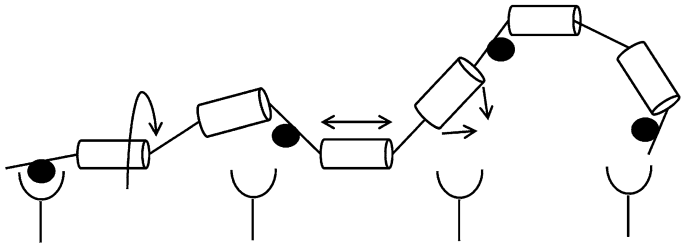


Fig. 14.7 Entropy losses from constraint of a flexible linker. *Circles* (●) represent ligand-binding sites, while cylinders represent rigid segments of a flexible linker. Arrows indicate degrees of freedom of movement for the polymer chain; each segment can undergo rotation (*left*), extension (*middle*), and bending (*right*)

receptors on a surface. On the simplest level, the longer and more flexible the linker is, the greater the entropic penalty paid for each subsequent binding event. To illustrate this concept, we expand our polymer chain model to include identical ligand-binding sites (represented by ●) interspersed along a chain of length $l \gg L_P$ (Fig. 14.7). Each chain segment has several degrees of freedom from the translational, rotational, and conformational contributions to entropy. Therefore, each binding event results in an entropy loss for the linker as well as for the ligand binding site and receptor. In Fig. 14.7, the first binding event on the left pays the majority of the entropic penalty and localizes the entire ligand in proximity to other receptors. Although subsequent binding interactions will also incur entropy losses for the linker and ligand-binding sites, these losses decrease for each additional binding event. If these entropic losses are less than the energy necessary to bring each monovalent binding site from solution to the receptor, the overall $\Delta G_{\text{multi},N}$ is still lower than the sum of N separate $\Delta G_{\bullet,\,\text{mono}}$ and there will be an avidity effect. However, if the linker is too elastic or too long, the overall entropic penalty for binding the entire ligand increases, as would $\Delta G_{\text{multi},N}$. In this case, avidity would decrease, resulting in a weaker binding affinity. If the overall entropic penalty increases to the point where $\Delta G_{\text{multi},N} = N\Delta G_{\bullet,\text{mono}}$, then no avidity effect will be observed.

In the case of a an idealized multivalent protein, there is no entropy loss upon the interaction of subsequent binding sites, and the apparent affinity is thus the theoretical maximum; that is, the linker is perfectly designed so that the first binding event essentially places all remaining binding sites in an optimal orientation relative to their respective binding partners. A classic example of using linkers that maximize avidity is the binding of trivalent vancomycin and trivalent D-Ala-D-Ala [37]. The monovalent affinity between the vancomycin and D-Ala-D-Ala monomers is ~ 1 μM; by trimerizing both receptor and ligand, an overall affinity of sub-fM binding was achieved, an increase of eleven orders of magnitude. The key to optimizing avidity in this case was selecting linkers rigid enough to minimize entropy loss during intermolecular binding.

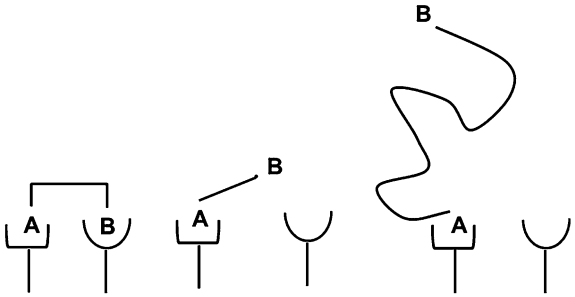


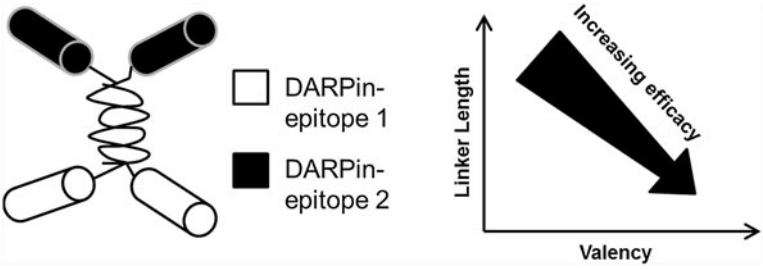
Fig. 14.8 Linker effects on binding. *Left*: Idealized case. Linker length and elasticity is theoretically optimal for constraining *B* in proximity to its receptor upon binding of *A*. *Middle*: Linker length is too short/inelastic, such that the limited volume sampled by *B* does not permit its binding to its receptor. *Right*: Linker length is too long/elastic such that the volume sampled by *B* is similar to free *B* in solution, resulting in negligible avidity effects

Despite this elegant example of a "perfect" multivalent interaction, in the majority of cases avidity effects will fall below the theoretical maximum. In practice, even with an optimized linker, entropic penalties can still occur. In the vancomycin/D-Ala-D-Ala example above, both interacting molecules were soluble and presented to each other as pre-formed trimers, minimizing the entropy loss upon binding compared with physiological protein–protein interactions that require conformational changes or receptor clustering to effectively bind. Linker design is ultimately a balance between elasticity and minimizing entropy loss. In cases where receptor spatial orientation is well characterized, a rigid linker that positions the binding sites at the exact distances between receptors is desirable. This arrangement minimizes the loss of entropy upon binding of each site and approaches the optimal affinity possible for the multivalent construct (Fig. 14.8, left). In contrast, a short and/or rigid linker that cannot span two receptors results in monovalent binding (Fig. 14.8, middle). Alternatively, the entropic costs conferred by a long, elastic linker (Fig. 14.8, right) can be large enough to where avidity effects are negligible. For more detailed information about linker effects on receptor binding, the reader is referred to texts from Krishnamurthy et al. [19] and Zhou [26].

A wide variety of linkers have been used for tethering multiple binding sites. While chemical linkers of varying composition have been used to conjugate proteins [38–40], challenges often arise with heterogeneous product formation and purification; thus, these linkers are often used to tether peptides and small molecules produced through synthetic methods. Peptide-based linkers, which can be expressed as genetic fusions between proteins, have generated great interest as alternatives [41]. Common elastic linkers include combinations of glycine and serine, while inelastic linkers tend to include conformationally constrained amino acids such as proline, or residues that form stable α-helices [36]. Natural protein domains have also been exploited as tethers and provide geometrical rigidity to restrain the binding sites of a multivalent protein [42]. The most well-studied

example in protein therapeutics is again the antibody. The two variable regions of an antibody are fused to a constant domain that fixes the spatial distance between the antigen-binding sites. Antibodies have therefore been used as robust molecular scaffolds to create more than 35 different fusion proteins, spanning a broad range of multivalent and multispecific protein architectures [8, 9, 43]. Several such antibodies have advanced to clinic trials, underscoring the adoption of multivalency and multispecificity in novel protein design [43]. More recently, multivalency and multispecificity have been engineered into single-domain proteins, providing a novel method to constrain the orientation and topological arrangement of binding sites [44–46].

Example 14.1: *Tuning Protein Valency and Linker Design for Improved Biological Potency.*



Epidermal growth factor receptor (EGFR) overexpression is associated with many cancers, thus there has been great interest in designing protein therapeutics that inhibit the receptor and its downstream cell-signaling pathways. In Boersma et al. [47], designed ankyrin repeat proteins (DARPins) were evolved to bind distinct EGFR epitopes with sub-nM affinities. The authors then used these engineered DARPins to create bispecific/bivalent, as well as bispecific/tetravalent fusions, with the goal of improving biological efficacy.

A trend of increased efficacy, as measured by cell growth inhibition, was observed with increased valency. Furthermore, the engineered molecules demonstrated varying degrees of inhibition in cell culture models depending on linker length, linker elasticity, and monomer orientation. Specifically, a bispecific/tetravalent DARPin fusion was created by tethering individual monomeric subunits to a rigid leucine zipper, via different elastic glycine or glycine-serine linkers (inset). In this study, the shortest linkers, namely Gly_4 and Gly_2Ser_2, resulted in multivalent proteins with the highest levels of biological inhibition. These results demonstrate that valency and linker length can be tuned to improve biological potency.

14.3 Tuning Selectivity Through Multivalency and Multispecificity

In Sect. 14.2, we introduced fundamental biophysical principles underlying protein–protein interactions and discussed how these concepts explain avidity effects in the case of multivalent proteins. The examples in the previous section used multivalency and multispecificity as tools to engineer proteins with higher affinity to a target of interest. We now consider protein therapeutics in a physiological context, with the critical question: How can we use the properties of multivalency and multispecificity to effectively distinguish between receptor targets that are associated with disease versus those that are necessary for normal biological processes? The goal of any protein therapeutic is to target specific cells and/or specific biological pathways involved in disease pathology. Protein therapeutics that bind a target receptor with high affinity and specificity can be effective in cases where the target receptor is expressed at high levels within a disease site, or if disease is caused by a mutated receptor that is distinct from the natural one. Challenges arise, however, when there is not a marked difference between "target" and "non-target" states. The inability of a protein therapeutic to distinguish between these two states can lead to undesired off-target effects and toxicity [48]. In this section, we will explore how target receptor selectivity can be increased with multivalent and multispecific proteins, namely by preferentially targeting diseased cells with higher numbers of receptors, or by targeting two different receptors on diseased cells (Fig 14.9).

First, we will define *specificity* and *selectivity* in the context of this chapter. Specificity refers to the ability of a protein therapeutic to distinguish between its target receptor and all other biomolecules; namely, by having a higher binding affinity to this target. Selectivity refers to the ability of a protein therapeutic to bind with greater affinity to target receptors on diseased cells. Mathematically, it can be represented as:

$$\text{Selectivity} = \frac{\#\text{ligand bound to target cells}}{\#\text{ligand bound to non-target cells}} \tag{14.18}$$

Specificity is usually required for, but does not guarantee, selectivity. Therapeutic efficacy is determined in part by selectivity, and whether a drug is able to carry out its intended function without off-target effects.

As stated above, the important criteria for an effective protein therapeutic is tight binding to target cells and weak to no binding to non-target cells. This translates to the expression: $K_{D,\text{target}} \ll K_{D,\text{non-target}}$, which should be familiar to readers as it is similar to our description of avidity. In Sect. 14.2.3, we defined parameters for avidity where any subsequent binding event for a multivalent protein has a stronger apparent affinity than that for a monovalent binding event ($K_{D,A}{}' \ll K_{D,A}$ and $K_{D,B}{}' \ll K_{D,B}$). When considering a protein therapeutic that has specificity toward receptors on both target and non-target cells, *to achieve selectivity for target cells, it must bind these cells with an apparent affinity*

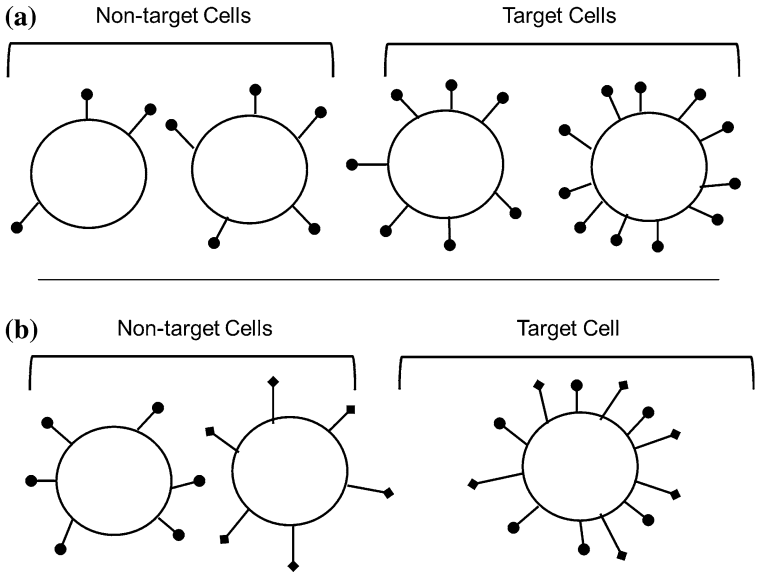


Fig. 14.9 Two scenarios that can be exploited with multivalent and multispecific proteins to preferentially distinguish between target and non-target cells. **a** Non-target and target cells both express the same receptors (●), with target cells expressing receptors at a slightly higher density. **b** Target cells express two receptor types abundant on non-target cells (●, ■). Non-target cells only exclusively express one receptor type

bolstered by avidity, while binding non-target cells at a weak, characteristically monovalent affinity. Thus, by engineering a protein therapeutic to be multivalent and/or multispecific, one can achieve this difference in apparent affinity. The optimal therapeutic needs to strike a delicate balance: while the overall affinity for the target cell should be strong, the individual affinities of each binding interaction must be relatively weak. This is the key to selectivity.

Using the scenarios in Fig. 14.9 as model systems, we will demonstrate how multivalency and multispecificity can lead to selectivity. More detailed analyses of these models can be found in Caplan and Rosca [49], which is our main source for this discussion. Each model will be complemented by an example from literature that uses the concepts of multivalency and multispecificity to achieve high selectivity in targeting a diseased state; these models can serve to inform the design of protein therapeutics with improved efficacy and safety profiles.

14.3.1 Model 1: Selectivity Based on Receptor Density

For an intuitive understanding of selectivity, we will analyze the behavior of monovalent, bivalent, and higher order multivalent ligands in a mixture of target and non-target cells that differ in receptor density (Fig. 14.10).

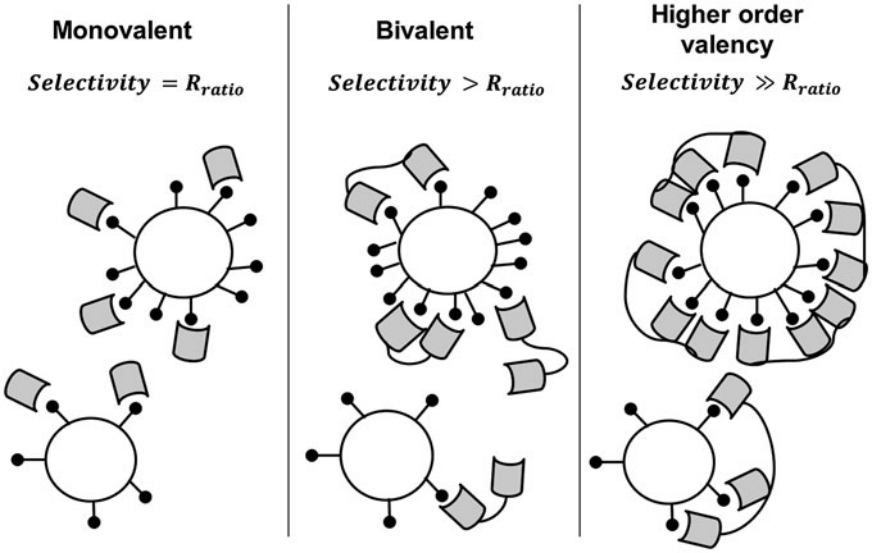


Fig. 14.10 Selectivity increases with valency. R_{ratio} is defined as the receptor density ratio of target over non-target cells. In this scenario, $R_{\text{ratio}} = 2$

Suppose diseased cells express twice as many receptors as normal cells, as in Fig. 14.10. Let us begin by considering the case of a monovalent ligand, as shown in the left panel. The affinity of the ligand for receptor is the same for both cell types, and thus, the number of bound ligands on the cell surface is directly proportional to the receptor density. In this example, the selectivity of the monovalent ligand is simply 2:1 for diseased cells to normal cells; to make a general statement, selectivity is equal to the receptor density ratio. Now, we will consider how a bivalent ligand would behave. The schematic in the middle panel suggests that selectivity is enhanced, and the relative number of bound ligands is greater than the receptor density ratio. We can understand this phenomenon from the principle of avidity. The first binding event has the same kinetics as that for the monovalent ligand, but the increase in C_{eff} of the second binding site dramatically drives further ligand–receptor association (Eq. 14.16). However, this increase in C_{eff} is useful for increasing the apparent affinity only when there is an appropriately large receptor density on the cell surface to participate in the second binding event. This means that for a bivalent ligand, the apparent K_D on cells with low receptor density is similar to that of the monovalent ligand. In contrast, on cells with high receptor density, avidity results in an apparent K_D value that is much tighter than that for the monovalent ligand. This difference in apparent affinity is the basis for a multivalent protein's selectivity for cells with higher receptor density. With this idea in mind, since greater valency can lead to greater avidity, the selectivity must also increase with valency as seen in the right panel. Thus, to design a selective protein, one must determine both the degree of valency necessary, as well as the

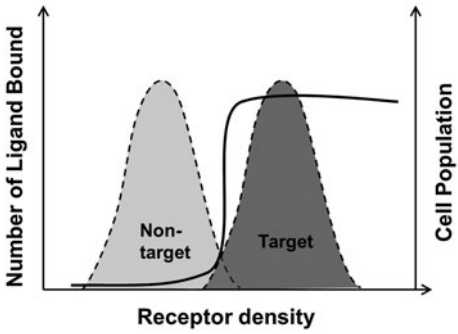


Fig. 14.11 Desired selectivity for distinguishing between target and non-target cells with slightly different receptor densities. The two peaks represent two overlapping cell populations. The *dark curve* is the number of ligand bound for a range of receptor densities

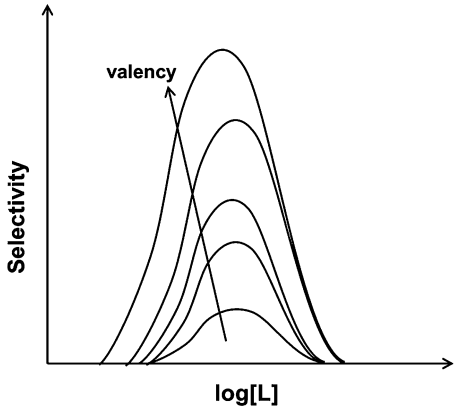


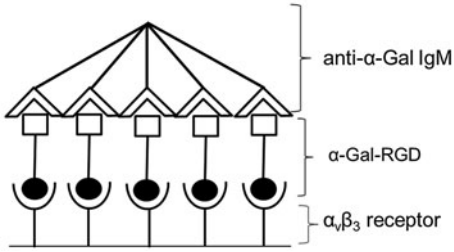
Fig. 14.12 Increased selectivity is observed between cell types with a fixed number of target and non-target receptor densities as ligand valency increases. Selectivity peaks at intermediate levels of ligand concentration and decreases at low and high concentrations of ligand. Figured adapted from Perelson [50]

optimal binding affinity of each individual binding domain. A general strategy for maximizing selectivity is to incorporate individual binding sites with weak affinity to eliminate substantial binding to non-target cells with low receptor density, while increasing valency such that strong affinity is achieved toward target cells with higher receptor density. Figure 14.11 provides a graphical representation of the selectivity depicted in Fig. 14.10 for a multivalent ligand. Note that even for cell types with slight differences in receptor densities, one can in principle design protein therapeutics to discriminate between target and non-target cells effectively.

At fixed target and non-target cell receptor densities, the selectivity phenomenon in Fig. 14.11 depends highly on ligand concentration. In Fig. 14.12, we see that the selectivity of multivalent ligands can be described by bell-shaped curves. In the case where ligand concentration is very low, selectivity is reduced since the ligands form very few complexes on both target and non-target cells. At intermediate levels of ligand concentration, selectivity reaches a peak and multivalent ligands will display enhanced selectivity, as they have more opportunities to form multiple binding interactions with receptors on the cell surface. Proteins with high order valency have increased apparent affinity to target cells compared with

non-target cells, which results in increased selectivity. As the ligand concentration increases, the selectivity is again reduced, as the ligand is in great excess and will bind receptors on both cell types primarily through monovalent interactions. In other words, a high number of ligand molecules are competing for a limited number of receptor sites, such that at equilibrium relatively few are able to bind multivalently to the cell surface.

Example 14.2: *Directing An Immune Response Against Cells that Express High Levels of $\alpha_v\beta_3$ Integrin.*



$\alpha_v\beta_3$ integrin is a cell surface receptor that is expressed at high levels on many tumor cells and their vasculature. In Carlson et al. [51], $\alpha_v\beta_3$ integrin served as a model receptor to test the selectivity of a tumor-targeting agent. In this study, a modular multispecific agent was constructed by conjugating a peptide, containing an integrin-binding Arg-Gly-Asp (RGD) motif, to a highly immunogenic trisaccharide Galα(1-3)Galβ(1-4)Glc (α-Gal). The α-Gal-RGD conjugate (inset) relies on the binding of pentameric human anti-α-Gal IgM to the α-Gal moiety to recruit the complement system and stimulate cell death. Anti-α-Gal IgM binds to α-Gal through weak multivalent interactions: each of its five binding sites has a K_D value in the μM range, while the overall apparent K_D to an array of α-Gal is in the fM range. Drawing from the model introduced above, the anti-α-Gal IgM should bind weakly to cells with a low $\alpha_v\beta_3$ integrin density, and strongly to cells with a high $\alpha_v\beta_3$ integrin density.

The results showed that the multivalent strategy of cell targeting was highly selective and discriminated between several cancer cell lines with varying densities of $\alpha_v\beta_3$ integrin. The α-Gal-RGD conjugate only induced death in cells with receptor numbers above a certain threshold; cells with receptor numbers below this threshold remained viable. While this study took advantage of the selectivity inherent in the immune system, one can imagine using similar concepts to design other multivalent proteins that mimic this recognition strategy.

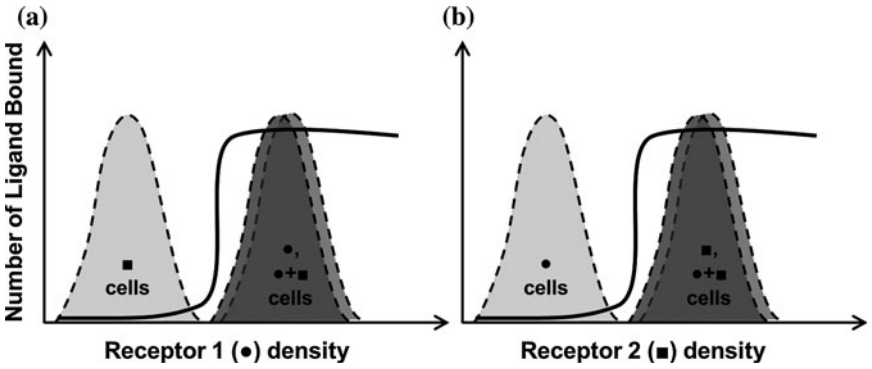


Fig. 14.13 Monospecific ligands from Model 1 do not discriminate between target cells (● + ■) and non-target cells (● only, ■ only). **a** Selectivity of a ligand specific for Receptor 1 (●). **b** Selectivity of a ligand specific for Receptor 2 (■)

14.3.2 Model 2: Specificity Based on Receptor Type

We now describe a second, more complex model where effective targeting is not achieved with the monospecific, multivalent proteins described above but instead requires a different strategy. Suppose we have three cell types, two normal and one diseased. The two normal cell types each express a different receptor (Receptor 1 or 2), while the diseased type expresses both receptor types (Fig. 14.9b). There is no difference in receptor density between the normal and diseased cell types for either receptor. If we create two monospecific, multivalent ligands, one that is specific for Receptor 1 and another that is specific for Receptor 2, either ligand will effectively bind the target cells, but will also have significant off-target binding effects (Fig. 14.13). It is clear that neither a monovalent nor a monospecific, multivalent ligand will confer selectivity in this scenario.

In this example, only a multispecific ligand will confer selectivity; it will bind with monovalent affinity to either of the normal cells and will exhibit substantially increased affinity for the diseased cells where multivalent ligand–receptor complexes are possible (Fig. 14.14). As with the first model in Fig. 14.12, a similar bell-shaped curve is seen in response to ligand concentration on both diseased and normal cells. Consequently, multispecific protein therapeutics are often necessary to effectively target one cell type over another and as such have generated great interest for protein-based drug design [52].

Example 14.3: *Bispecific Antibody Fragments Optimized for Tumor Targeting*

In this example, the target cells express two particular receptors that are also individually abundant in non-target cells. Robinson et al. [53] engineered a bi-specific single-chain Fv antibody (bs-scFv) whose variable regions bind to ErbB3 and human epidermal growth factor 2 (HER2) with affinities of 160 and 1.6 nM, respectively, as measured by surface plasmon resonance. Despite this measured ErbB3 affinity, the bs-scFv did not exhibit appreciable binding to an $ErbB3^{+-}$ $HER2^{-}$ cell line, even at concentrations of 1 μM. The selectivity of the bs-scFv to $ErbB3^{+}HER2^{+}$ cells over $ErbB3^{-}HER2^{+}$ cells was determined using flow cytometry, with the goal of binding to cells expressing both receptors while excluding cells that express one receptor. The results mirrored our description of Model 2: at high antibody concentration, there was no discrimination between the two cell types, most likely due to monovalent binding of the bs-scFv. As the concentration of bs-scFv decreased, however, the selectivity gradually increased. At 100 pM, there were ten bound $ErbB3^{+}HER2^{+}$ cells for every one $ErbB3^{--}$ $HER2^{+}$ cell bound. These data were confirmed in an in vivo experiment, where the bs-scFv accumulated in tumors expressing both receptors to a significantly greater extent compared with tumors expressing only one of the receptors. Thus, a combination of multispecificity and low-affinity monovalent interactions between ligand and receptor led to optimal targeting of a particular cancer cell type.

The above examples describe several points of consideration for improving the selectivity of a protein therapeutic. Ideally, the target cell should be distinguished from non-target cells in some way, typically by a unique receptor fingerprint. Multivalency or multispecificity can be exploited to improve selectively; however, receptor expression patterns and affinities of individual binding sites should be carefully considered to optimize selectivity for target versus non-target cells. One can often turn to nature for inspiration when designing protein therapeutics based on these concepts; several reviews provide good examples where multivalency and multispecificity are key to achieving a desired biological effect [12, 18].

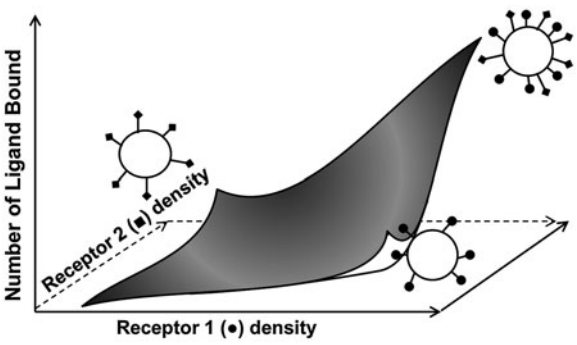


Fig. 14.14 Three-dimensional portrayal of multispecific ligand selectivity over a range of receptor densities. The multispecific ligand will bind with monovalent affinity to non-target cells expressing individual receptors (●) and (■), and with multivalent affinity to target cells that express both receptor types (● + ■)

14.4 Biological Principles for Multivalent and Multispecific Protein Design

In Sect. 14.2, we saw that avidity effects from multivalency increase the overall affinity of an interaction, and in Sect. 14.3, we saw that multivalency and multispecificity can be used to confer selectivity. We will now discuss ways in which these concepts can be partnered with knowledge of underlying biological principles to design effective protein therapeutics. Some questions to consider at the outset are the following:

1. What is the desired biological outcome, and what input signals should be targeted?
2. What is the goal for the multivalent or multispecific protein? To increase affinity? To increase selectivity? Or to control the oligomerization state of the target?
3. Is affinity and selectivity enough to counter the effects of competing ligands and the complexity of biochemical pathways?

In practice, it is sometimes difficult to predict the physiological response elicited by a protein therapeutic. In particular, the biological consequences of simultaneously modulating multiple biomedical targets is often unknown and can have unintended effects [54]. However, examples of carefully designed multivalent and multispecific proteins that have achieved a desired therapeutic outcome are growing [55], with several candidates moving through the clinical pipeline [43]. In this section, we will present three complex biological problems and provide examples of how multivalent and multispecific protein therapeutics can be used to address them.

14.4.1 Complexity of Cell-Signaling Pathways: Receptor Crosstalk and Biological Redundancies

Physiological responses are regulated by a complex network of cell-signaling pathways. Receptor crosstalk, where multiple biochemical inputs modulate the activity of one another (Fig. 14.15), occurs in both healthy and diseased states [56–59]. In the context of oncology, receptor crosstalk has limited the efficacy of traditional monospecific protein inhibitors, as alternative cell-signaling networks can compensate for the effects of the inhibited pathway [60–62]. Nature has also evolved mechanisms of biological redundancy where multiple ligands and receptors carry out similar effects, thus inhibiting only one ligand/receptor interaction with a monospecific protein may limit therapeutic efficacy [59]. In contrast, multiple cell-signaling pathways often need to be engaged to stimulate effective tissue regeneration, which is difficult to achieve with a monospecific protein [63].

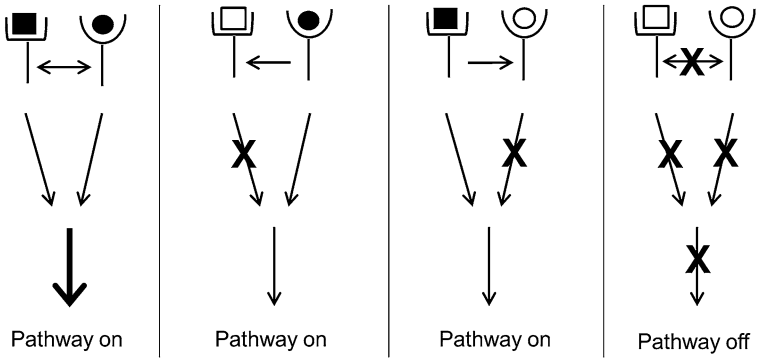
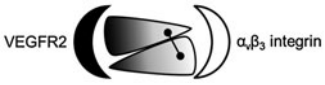


Fig. 14.15 Schematics representing receptor crosstalk. *Black-filled shapes* represent agonists (■, ●) while *white-filled shapes* represent antagonists (□, ○). Two receptors may exhibit crosstalk by stimulating the same pathway in parallel, or by affecting each other. Targeting one receptor (second, third panels) incompletely effects the pathway. Only by stimulating or antagonizing both receptors (first, fourth panels) is the pathway completely activated or inhibited

Receptor crosstalk can be achieved through several mechanisms: cells may alter their expression of non-target receptors, stimulating the target pathway through alternate means, or non-target receptors may amplify or inhibit the signaling of the target receptor to the point where even a high fraction of bound therapeutic does not result in a significant physiological effect [64] (Fig. 14.15). Thus, mechanisms for modulating the effects of multiple receptors are often sought to achieve increased control over a biological system. Accordingly, combination therapy can be more efficacious compared to treatment with individual monospecific agents, particularly in oncology indications [64, 65]. However, potential drawbacks to combination therapy are increased risk of toxicity from off-target effects, and increased cost [54, 65]. Multispecific protein therapeutics, in which each component binding site interacts with and inhibits a distinct receptor, have generated much interest for addressing these concerns. Simultaneous inhibition or stimulation of pathway inputs can greatly improve efficacy through additive or synergistic effects, often at lower doses compared with monospecific agents [11, 66].

Example 14.4: *A Bispecific Protein is More Potent Compared to Monospecific Agents*



VEGFR2 and $\alpha_v\beta_3$ integrin have been implicated in tumor-associated blood vessel formation, a process known as angiogenesis. Due to compelling evidence for crosstalk between VEGFR2 and $\alpha_v\beta_3$ integrin and their cell-signaling pathways

[67], there has been great interest in developing multispecific proteins that inhibit both of these receptors, with the goal of developing therapeutics that can effectively block tumor neovascularization.

Papo et al. [45] used the native VEGF ligand as a molecular scaffold to create a bispecific protein that bound to both VEGFR2 and $\alpha_v\beta_3$ integrin with high affinity. The receptor-binding sites of a VEGF homodimer are located on opposite poles of the protein. To create the bispecific molecule, VEGFR2 binding at one end of the protein was abolished and replaced with a new epitope that conferred binding to $\alpha_v\beta_3$ integrin (inset). Monospecific variants of these proteins that bound VEGFR2 or $\alpha_v\beta_3$ integrin alone were created for direct comparison. The bispecific protein was able to simultaneously engage both VEGFR2 and $\alpha_v\beta_3$ integrin, and more strongly inhibited receptor-mediated angiogenic processes *in vitro* and *in vivo* compared to monospecific agents, which were only marginally effective. These results demonstrate that antagonizing two receptors using a multispecific protein can have synergistic or additive effects on biological potency.

14.4.2 Receptor Trafficking

Attenuation of cell signaling can occur through ligand-induced receptor internalization or receptor desensitization [68]. Consequently, protein therapeutics that alter cell surface receptor densities have the potential to affect disease pathologies associated with receptor-mediated cell-signaling pathways. An intimate knowledge of receptor internalization and trafficking dynamics is required to design protein therapeutics that achieve this goal. Figure 14.16a shows a typical model for receptor trafficking. Upon activation or clustering, a receptor is endocytosed into the cell where it is sorted either for degradation or recycling back to the cell surface. It is the relative rates of synthesis, endocytosis, recycling, and degradation that determine the steady-state receptor density on the cell surface. For a detailed analysis regarding mathematical models for receptor trafficking refer to Lauffenburger and Linderman [69]. Each of these rates is a potential target that could be altered to regulate cell signaling through downstream pathways. For example, if inhibition of receptor activity is desired, one might engineer a ligand that increases endocytosis and degradation rates. If cell stimulation is desired, then one might engineer a ligand that increases the recycling rate (Fig. 14.16b).

Thermodynamic principles play a large role in regulating receptor trafficking, by directing receptor clustering and cell-signaling events. A major consideration is therefore how to geometrically constrain a certain density of receptor (which requires a large loss of entropy), while maintaining high affinity to the target cell (low ΔG of binding). A multivalent and multispecific protein therapeutic works particularly well in meeting these criteria. Optimization of linkers and binding site orientation within these protein fusions can drastically reduce the entropy of forming multiple ligand/receptor complexes on the cell surface.

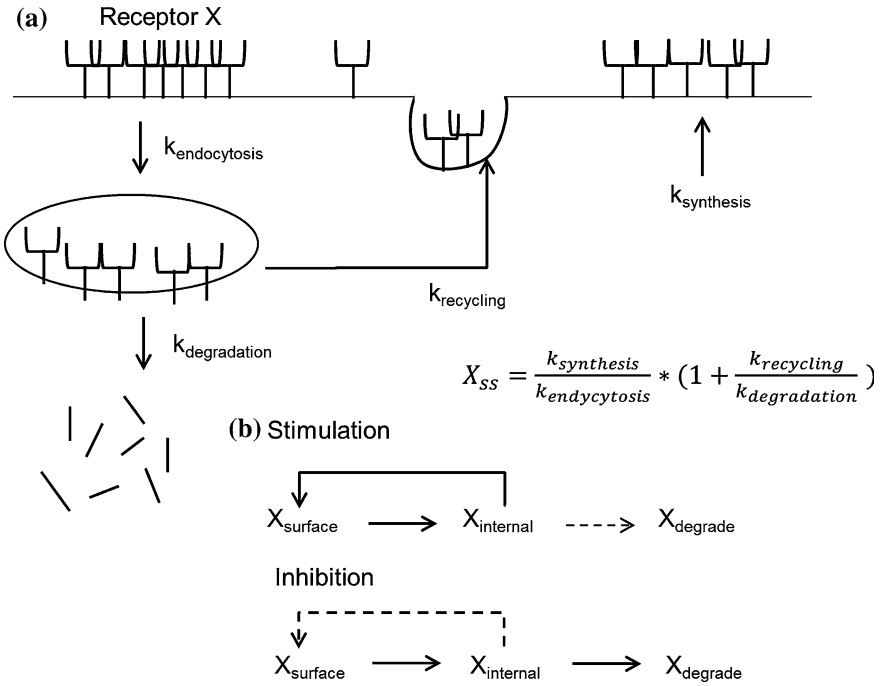
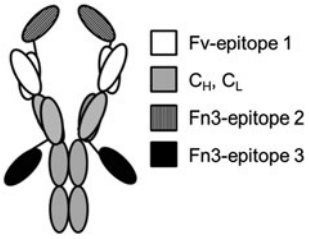


Fig. 14.16 Schematic of receptor trafficking. **a** Trafficking of receptor *X* can be summed up by the processes of synthesis, endocytosis, degradation and recycling. X_{ss} is the steady-state concentration of *X* on the cell surface. **b** Strategies for stimulating or inhibiting receptor *X*. Solid arrows indicate increase in rate, dashed arrows indicated a decrease in rate. A mathematical description of the concepts illustrated here is described in Lauffenburger and Linderman [69]

Example 14.5: *Multivalent and Multispecific Therapeutic Proteins That Induce Receptor Clustering and Downregulation*



Multivalent and multispecific proteins targeting different epitopes of the same receptor can have an inhibitory effect [70], with the notion that receptor clustering through noncompetitive binding increases internalization and/or degradation rates, resulting in signal attenuation. Recently, Spangler et al. [71] created a hexavalent, trispecific protein that ablated cell signaling through ligand competition, receptor

clustering, and recruitment of immune system effector functions. Through competitive and noncompetitive receptor binding, affinity was enhanced and the target receptors were oriented in a specific spatial arrangement to promote receptor downregulation. Toward this goal, multivalent/multispecific constructs were created by linking an EGFR-specific antibody (cetuximab) to two engineered protein domains (Fn3) that bind distinct EGFR epitopes. Constructs with Fn3 domains attached at various locations on the antibody were tested for their ability to induce EGFR downregulation. A fusion protein which has one EGFR-specific Fn3 domain linked to the N-terminus of the heavy chain and another to the C-terminus of the light chain (inset) was shown to have optimal efficacy and induced robust EGFR clustering and internalization, consequently inhibiting downstream signaling events. Interestingly, molecules with the same valencies and specificities, but different orientations, were less effective; the superiority of a particular topology underscores the relevance of antibody fusion geometry in eliciting optimum receptor downregulation. In addition, this hexavalent, trispecific protein demonstrated a remarkable ability to inhibit tumor growth in cetuximab-resistant BRAF and KRAS mutant cancers. Finally, it was shown that the hexavalent, trispecific fusion protein downregulates EGFR through both increased endocytosis and decreased recycling. In comparison, combination monospecific therapy did not increase endocytosis rates above baseline levels [72]. These experiments showed that the topological arrangement and orientation of receptor-binding domains strongly influenced apparent binding affinity, as well as the observed biological effects.

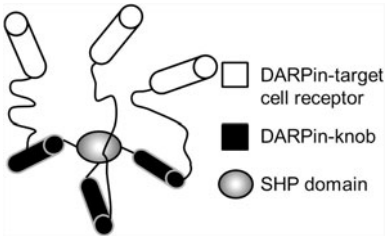
14.4.3 Multispecific Proteins for Recruitment and Delivery Applications

So far, we have seen applications where multispecific proteins target receptors on the same cell surface. In medicine, there are many instances in which two different cells, or a cell and a therapeutic moiety, need to be brought in close proximity for a therapeutic effect (Fig. 14.1). In these cases, the multispecific protein is an adaptor, rather than the actual therapeutic agent. Prime examples are bispecific antibody fragments engineered to recruit immune system effector cells to tumors [73]. In these constructs, one arm of the bispecific binds an overexpressed receptor on the tumor surface, while the other arm binds a receptor unique to the effector cell. More recently, there have been examples of adaptor bispecific proteins that bring viruses or nanoparticles with a gene or drug payload to diseased cells [74, 75], as well as bispecific proteins that bridge stem cells to injured tissue during tissue regeneration [76].

Cell recruitment and therapeutic delivery present interesting applications for multispecific proteins. One can consider these scenarios as a series of smaller protein engineering problems where the protein ligand requires separate affinity

and selectivity optimization for each of its binding targets. Ineffective interactions with target cells or therapeutic moieties may result in low efficacy or off-target effects. In addition, the linker that bridges the components of a multispecific protein adaptor may be instrumental in dictating the relative spatial distance and orientation of the target cell or therapeutic entity necessary to promote the desired biological effect. Thus, even if the multispecific protein does not directly serve as a therapeutic agent and instead acts as an adaptor, its design still needs to follow thermodynamic and kinetic principles as outline above.

Example 14.6: *Engineered Hexavalent, Bispecific Proteins for Targeted Gene Delivery*



Targeted gene therapy has been a long-standing goal for a number of genetic diseases. Dreier et al. [77] developed a modular, multispecific protein adaptor that links adenoviruses (gene carriers) to target cells. For drug/gene delivery applications, the essential characteristic of a protein adaptor is extremely high affinity to the therapeutic payload, in this case the adenovirus. Specifically, the dissociation rate as determined by k_{off} of the adaptor should be slower than the time needed to deliver the therapeutic to target cells. This is important for both toxicity and efficacy, as without an attached targeting moiety the therapeutic may act indiscriminately on non-target cells. To accomplish this goal, the authors used the capsid-stabilizing protein of lambdoid phage 21 (SHP domain) to create a trimer of a designed ankyrin repeat protein (DARPin), evolved to bind a receptor recognition knob protein on adenovirus. The DARPin trimer was then genetically linked to three identical DARPins, engineered to bind receptor targets expressed on cancer cells (inset). Adenovirus complexed through the multispecific DARPin adaptor was tested for transduction efficiency against several target cancer cell lines.

First, SHP domain-mediated trimeric adaptors outperformed monovalent and bivalent adaptor constructs in binding adenovirus knob protein. This suggests that the additional valency of the trimer presented avidity effects that allowed strong binding between adaptor and adenovirus. Second, spatial arrangement as well as valency contributed to affinity. In one experiment, an adaptor construct with linearly arranged knob-specific DARPins dissociated from adenovirus within one day and could not initiate target cell transduction. In contrast, a construct where the knob-specific DARPins formed a triangular arrangement around the central SHP

domain was able to stay attached to adenovirus and maintain unchanged transduction efficiency for at least ten days. In this case, entropic effects from the linkers had a large effect on avidity and proved to be an integral part of protein design. In final experiments, the hexavalent, bispecific adaptor successfully led to selective transduction of cell lines depending on the specificity of the receptor-binding DARPins attached to the knob-binding trimer. As this study shows, an effective adaptor for targeted gene delivery can be created by tuning valency and binding orientation.

14.5 Conclusion

In this chapter, we present the biophysical principles underlying binding affinity, avidity, and selectivity and provide examples of their considerations for the design and engineering of protein therapeutics. Nature has long exploited the concepts of multivalency and multispecificity for optimal control of biological processes. Inspired by these examples, biomolecular engineers have begun to create elegant protein-based molecular architectures that more effectively address complex medical challenges.

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