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PERSPECTIVE

Antibodies to Modulate Surface Receptor Systems Are Often Bivalent and Must Compete in a Two-Dimensional Cell Contact Region

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Mechanistic models have been developed to calibrate to and predict pharmacodynamic (PD) responses for therapeutic antibodies that modulate cell surface molecules. To accurately reflect *in vivo* antibody–receptor interactions and predict receptor occupancy and proximal PD, a thorough accounting of biophysical principals for bivalent antibodies and surface-constrained receptor–ligand systems needs to be incorporated. We provide salient biophysical considerations for cell-surface receptor–ligand interactions, including immune checkpoints, that necessitate model-guided, quantitative analysis of experimental results.

BIVALENCY AND A STRATEGY FOR DETERMINING ANTIBODY RATE CONSTANTS

Unless designed otherwise, many antibodies are intrinsically bivalent, and it is well-recognized that apparent binding behavior is impacted by antigen surface density.[1] Integrated experimental and modeling methods using both surface plasmon resonance–based kinetic measures and cell-binding data have been used to account for the kinetics of individual receptors, their apparent binding affinity, and more important their cross-linking behavior.[1] However, to extrapolate and assess binding competition with other molecules on the surface of cells, we recommend measuring the absolute amount of antibody bound per cell and extracting a two-dimensional cross-linking rate that also accounts for surface area. One possibility we have tested is to modify previously reported cell-binding protocols to use primary labeled antibodies,[1] quantifying bound antibody with flow cytometry, and calibrating with detection beads with a known number of antibody-binding sites. The cross-linking rate can therefore be quantified by modeling the experimental binding assay, with the cross-linking rate reported as a two-dimensional association rate with dimensions of surface area per molecule per unit time.

Our proposal builds on previous models for determining receptor–antibody interactions,[1] with a caveat that we parameterize our system explicitly in two dimensions. First, we recommend determining true monovalent on and off rates for a monovalent antibody interaction with antigen, either with suitable antibody fragments or monovalent antigen constructs. For the purpose of this discussion, we use the terms *receptor* and *antigen* interchangeably. Next, we recommend binding titration experiments for the antibody, with increasing concentrations of antibody. Here, rather than assuming an equilibrium analysis, we advocate for simulating the incubation conditions and using the quantitative experimental binding data, with bound molecular site densities calculated at the end of each incubation, for model fitting. Note the incubation may not necessarily reach equilibrium over an hour, even with a fast monovalent association rate. If measuring association at 37°C, steps can be taken to minimize internalization if needed, such as treatment with sodium azide.

Ordinary differential equations describing the mass balance for solution phase antibody binding, monovalently bound complex, and bivalently bound complex are (parameters and state variables are detailed in **Table 1** and select variables are shown in **Figure 1a** for clarity):

$$\frac{\mathrm{d}X_A}{\mathrm{d}t} = -2k_a\frac{X_A}{V_W}\left(X_{\mathrm{RT}} - X_{C1} - 2X_{C2}\right) + k_r X_{C1} \tag{1}$$

$$\begin{aligned}\frac{\mathrm{d}X_{C1}}{\mathrm{d}t} &= 2k_a\frac{X_A}{V_W}(X_{\mathrm{RT}} - X_{C1} - 2X_{C2}) - k_r X_{C1} \\ &\quad - k_{\mathrm{xa}} N_{\mathrm{nmole}} \frac{X_{C1}}{4\pi r_{\mathrm{cell}}^2 N_{\mathrm{cell}}}\left(X_{\mathrm{RT}} - X_{C1} - 2X_{C2}\right) + 2k_r X_{C2}\end{aligned} \tag{2}$$

$$\frac{\mathrm{d}X_{C2}}{\mathrm{d}t} = k_{\mathrm{xa}} N_{\mathrm{nmole}} \frac{X_{C1}}{4\pi r_{\mathrm{cell}}^2 N_{\mathrm{cell}}}\left(X_{\mathrm{RT}} - X_{C1} - 2X_{C2}\right) - 2k_r X_{C2} \tag{3}$$

The total bound antibody can be calculated from:

$$X_B = X_{C1} + X_{C2} \tag{4}$$

Note that we employ quantities rather than concentrations as the state variables. Although not strictly necessary for this small model and fixed volume system, this strategy helps to avoid errors in systems with variable control volumes, such as growing tumors, and conceptually simplifies modeling with binding across interfaces. Also note that an internalization term has not been included here, but it is relatively straightforward to include with additional supporting data if needed.

The surface density of bound antibody, which can be fit to experimental measures, can be calculated from:

$$B = \frac{N_{\mathrm{nmole}} X_{\mathrm{B}}}{4\pi r_{\mathrm{cell}}^2 N_{\mathrm{cell}}} \tag{5}$$

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Table 1 Model state variables and parameters

Symbol	Description	Suggested units
k_a	Free antibody association rate with free antigen	nM^{-1} day^{-1}
k_r	Dissociation rate of monovalent antibody–antigen complexes	day^{-1}
k_{xa}	Cross-linking rate for antibody–antigen complexes	μm^2 $molecule^{-1}$ day^{-1}
N_{cell}	Number of cells in the assay well (often around 1×10^5 for avidity experiments)	number
N_{nmole}	A conversion factor from nanomole to molecules (6.022×10^{14})	molecule $nanomole^{-1}$
r_{cell}	Assumed cell radius	μm
V_w	Volume of the assay well	L
X_A	Amount of free antibody	nanomole
X_B	Amount of bound antibody	nanomole
X_{C1}	Amount of single antigen and antibody complex on the cell surface	nanomole
X_{C2}	Amount of double antigen and antibody complex on the cell surface	nanomole
X_{RT}	Total amount of antigen on the cell surface	nanomole
B	Density of total antibody bound on the cell surface	molecule μm^{-2}
σ	Confinement length	nanometer
$k_{a,lgnd}$	Solution phase association rate of receptor-ligand complexes	$nanomolar^{-1}$ day^{-1}
$k_{xa,lgnd}$	Two-dimensional association rate of receptor-ligand complexes	μm^2 $molecule^{-1}$ day^{-1}
$V_{convert}$	Volumetric conversion factor (1×10^{-18})	L nm^{-1} μm^{-2}
f_v	Vascular fraction for the tumor	dimensionless
f_{ICF}	Fraction of the nonvascular tumor volume that is intracellular	dimensionless
$f_{ECM,A}$	Fraction of the interstitial tumor volume that is not accessible to antibody due to extracellular matrix	dimensionless
$k_{in,DAg}$	Rate constant for antigen molecules to diffuse into the synapse	day^{-1}
$k_{out,DAg}$	Rate for antigen diffusing out of the synapse	day^{-1}
$Q_{prod,contact}$	Production rate of antigen for cells in contact	molecule day^{-1}
k_{sdeg}	Surface antigen degradation rate	day^{-1}
$k_{xr,lgnd}$	Dissociation rate of receptor–ligand complexes	day^{-1}
S_{syn}	Total surface area directly involved in cell–cell contact in the tumor (synapse), same on antigen-expressing or ligand-expressing cells	μm^2
$S_{Agnonsyn}$	Surface area on antigen-expressing cells in the tumor that are engaged in cell–cell contacts, but this portion of the surface is not directly involved in the synapse	μm^2
V_t	Total tumor volume	L
X_{At}	Amount of free antibody in the tumor	nanomole
X_{Agsyn}	Amount of free antigen in cellular synapses	nanomole
$X_{Agnonsyn}$	Amount of free antigen on contacting cells but outside of synapses	nanomole
X_{C1syn}	Amount of single-antigen antibody complexes in cellular synapses	nanomole
X_{C2syn}	Amount of double-antigen antibody complexes in cellular synapses	nanomole
$X_{lgndsyn}$	Amount of free ligand in cellular synapses	nanomole
$X_{CAglgnd}$	Amount of antigen–ligand complexes in cellular synapses	nanomole

We obtain initial estimates for the monovalent association and dissociation rates from surface plasmon resonance and then we simulate each incubation of fixed duration with the minimodel. The cross-linking rate and total receptor density are obtained by fitting to data across incubation concentrations. Fitting a statistical model, such as a nonlinear mixed effects model, can enable an estimate of variability in parameters such as receptor site densities. A single hypothetical data set and resulting fitted curve from fitting and simulations of bound antibody after 1 hour of incubation are shown in **Figure 1b** for clarity. Care must be taken with antibodies that exhibit fast monovalent dissociation kinetics, which may create difficulties measuring monovalent portions of the binding curve well if quick fixation is not an option. To better assess these issues, experimental checks coupled with simulation, such as observing dissociation kinetics of bound antibody preattached to cells with a pulse-chase incubation, can serve as additional verification for estimated cell-binding kinetic parameters.

SURFACE CONFINEMENT

Unlike soluble factor binding, surface receptor–ligand systems are not free to interact in three dimensions. For many immune interactions of clinical interest, T cells contact antigen-presenting cells or target cells, and receptors on T cells often bind to ligands on an opposing cell surface. The kinetic effects of this confinement have been demonstrated experimentally for receptor–ligand systems salient to immunology and immuno-oncology, and strikingly this cell-to-cell contact surface interaction has been reported to be confined to 3 nanometers in some cases,[2,3] with variation reported for different proteins that normally localize to the peripheral or central supramolecular activation complexes. Mathematically we may treat this confinement effect as enhancing the rate of bond formation, as has been done for previous systems analysis of immune checkpoint ligation based on biophysical considerations[4]; that is,

$$k_{xa,lgnd} = \frac{k_{a,lgnd}}{\sigma N_{nmole} V_{convert}} \tag{6}$$

The two-dimensional binding rate for ligand, $k_{xa,lgnd}$, increases with decreasing confinement length, σ, for a given three-dimensional association rate, $k_{a,lgnd}$. Additional information on the conversion factors and units is provided in **Table 1**. Cell–cell interactions therefore have the capability of

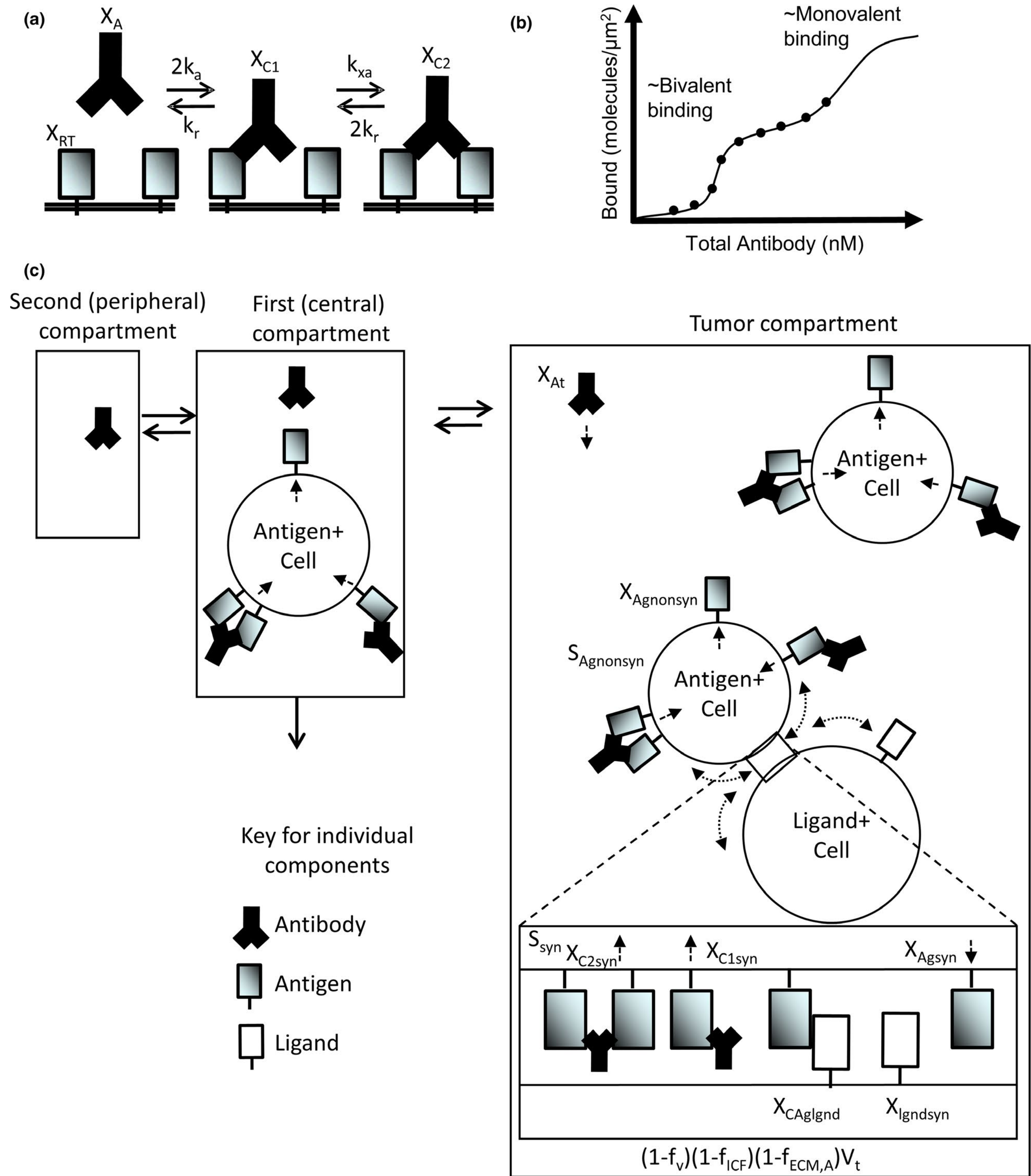

Figure 1 Illustration of models. (**a**) Cartoon of antibody-binding minimodel depicting monovalent antibody, first bond formation, and cross-linking. The model variables are detailed in **Table 1**. (**b**) Illustrative curve depicting the result of a typical data fitting. The dots are hypothetical data points that can be measured by flow cytometry experiments with paired calibration beads, and the fit curves are the output of simulations explicitly modeling the incubation to obtain parameter estimates for the cross-linking rate and total site density. Note that different regions and characteristics of the curve are differentially sensitive and best able to report different underlying biophysical parameters, such as the cross-linking, monovalent association rates, and total receptor site densities. (**c**) Illustration of a bigger model incorporating bivalent antibody–antigen interactions and competition for ligand. The model includes a two-compartment pharmacokinetic model, antibody binding to receptors in the blood and tumor, transport to the tumor, and accounts for antigen-expressing cells in the tumor that are both noncontacting as well as contacting other cells in the tumor that can engage in receptor–ligand interactions. The species detailed in the figure are involved directly with the antigen in the contact region as described in Eq. 7.

converting relatively weak solution receptor–ligand interactions into strong competing factors that should be evaluated in the context of an immune synapse when being pharmacologically modified. Furthermore, we can divide cell surfaces into contact and noncontact regions and account for diffusion between these regions when modeling the interaction between cell surface molecules. Agrawal and Linderman[5] demonstrated the importance of accounting for locally high concentrations of ligands in modeling the interaction between major histocompatibility complex and T cell receptors between helper T lymphocytes and antigen presenting cells. Additional surface receptors, including checkpoint molecules and ligands, may diffuse into the contact region.

RECEPTOR OCCUPANCY IS NOT A GOOD SURROGATE FOR TARGET MODULATION

The ratio of the available surface receptor bound to antibody vs. total receptor on the surface of cells, or the receptor occupancy with antibody, has been proposed as one metric to assess target binding and modulation. Modeling of the receptor occupancy with antibody can also incorporate the theoretical considerations of bivalency and ligand competition we have emphasized. However, it is important to note that receptor occupancy is a fractional measure of receptor available that is bound to antibody. A mechanistic consideration of signal transduction suggests that modulation of the number of target receptors per cell engaged in signaling is an important consideration for analysis. For example, in the case of checkpoints, cells may modulate negative signaling by upregulating the expression of checkpoint molecules. When checkpoint molecules are expressed at a higher level per cell, a larger fractional receptor occupancy by the antibody would be needed to achieve a similar number of receptors engaged in negative signaling and effective target modulation. The number of receptors per cell free to engage or engaged with endogenous ligands are therefore additional, alternate metrics worth exploring for extrapolative predictions with inhibitory receptors. The next section presents one strategy for how receptor–ligand engagement can also be incorporated with antibody binding in an immunological synapse.

THERAPEUTIC ANTIBODY AND LIGAND BINDING IN IMMUNO-ONCOLOGY

Once appropriate data are generated to support the estimation of cross-linking rates and site densities, as well as other processes such as internalization or shedding if needed, a model accounting for interactions in the tissue microenvironment can help to assess the impact of receptor–ligand interactions in the context of confinement and dosing. For the case of immuno-oncology, where the tumor is a site of interest, a representative cartoon of a model accounting for contacting and noncontacting cells expressing antigen as well as surface ligand that monovalently interacts with the antigen is shown in **Figure 1c**. In addition to a two-compartment pharmacokinetic model, we employ a mechanistic tumor transport model.[6,7] We wish to draw attention to an equation that could be used to describe the binding of monovalent receptor in the model while accounting for competitive ligand binding, cross-linking, and diffusion from the synapse region (parameters and state variables are detailed in **Table 1**):

$$\begin{aligned}\frac{dX_{\mathrm{Agsyn}}}{dt} = &-\Bigg(2k_a\frac{X_{\mathrm{At}}}{(1-f_v)(1-f_{\mathrm{ICF}})(1-f_{\mathrm{ECM,A}})V_T}+k_{xa}N_{\mathrm{nmole}}\frac{X_{\mathrm{C1syn}}}{S_{\mathrm{syn}}}\\ &+k_{\mathrm{xa,lgnd}}N_{\mathrm{nmole}}\frac{X_{\mathrm{lgndsyn}}}{S_{\mathrm{syn}}}+k_{\mathrm{out,DAg}}\Bigg)X_{\mathrm{Agsyn}}+k_rX_{\mathrm{C1syn}}\\ &+2k_rX_{\mathrm{C2syn}}+k_{\mathrm{xr,lgnd}}X_{\mathrm{CAglgnd}}+k_{\mathrm{in,DAg}}X_{\mathrm{Agnonsyn}}\\ &+\left(\frac{S_{\mathrm{syn}}}{S_{\mathrm{syn}}+S_{\mathrm{Agnonsyn}}}Q_{\mathrm{prod,contact}}-k_{\mathrm{sdeg}}X_{\mathrm{Agsyn}}\right)\end{aligned} \quad (7)$$

This equation draws attention to the role of cross-linking and competition between antibodies and ligands that occurs on the two-dimensional cell surface. The two-dimensional ligand binding, here with rate $k_{\mathrm{xa,lgnd}}$, establishes competition for the receptor binding to soluble antibody and cross-linking in two dimensions, with rates k_a and k_{xa}, respectively. Note that two-dimensional reaction formulations are not unique to modeling the disruption of checkpoint interactions. For example, agonistic antibodies may cross-link surface receptors, and bispecific T cell engaging antibodies may link T cell receptors with contacting target cell antigens in two dimensions on opposing cell surfaces. We have found ligand competition effects to be relevant when targeting proteins that can engage in synapses, especially as ligand densities increase, as assessed with tools such as multivariable sensitivity analysis that are established and available in quantitative systems pharmacology software.[8] We have applied a similar experimental and multimodel strategy to develop analyses to support both in-house decisions and regulatory interactions.[9] The last term describes the net rate of production and degradation of antigen, which may be simplified based on available data or further refined based on an antigen internalization, degradation, synthesis, and trafficking model. A thorough description of Eq. 7 is given in the **Supplementary Material**.

CONCLUSIONS

Although numerous models have been proposed to quantify the binding of immune checkpoint inhibitors,[10] the field will benefit from better characterization of antibodies, the receptor–ligand systems they are designed to modulate, and quantitatively reporting additional key biophysical properties. We posit that careful assay development and utilization of mechanistic model-based analyses of nonclinical data, here especially antibody kinetics and simple *in vitro* measures with isolated human cell types, will be broadly beneficial toward the development of systems models that more critically evaluate key receptor–proximal factors contributing to dose and exposure response.

Supporting Information. Supplementary information accompanies this paper on the *CPT: Pharmacometrics & Systems Pharmacology* website (www.psp-journal.com).

Supplementary Material S1.

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