Prediction of biomolecule kinetics using physics-based Brownian dynamics to data-driven machine learning methods

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Abstract

We present a comprehensive review of biomolecular binding kinetics and their modeling via Brownian dynamics simulations, with particular emphasis on enzyme–substrate interactions in cellular environments. We outline the theoretical foundations of Brownian dynamics (BD) and its application to modeling association and dissociation processes in both homogeneous and heterogeneous media. We further examine the role of BD in relation to emerging machine learning (ML) approaches for directly predicting binding kinetics. Finally, we propose that BD simulations can serve as a critical bridge between molecular-scale models and continuum, cell-level descriptions, offering a pathway toward a multiscale understanding of in vivo kinetic phenomena.

1 The Enzyme-Substrate Interaction in Biological Systems

1.1 The importance of enzyme kinetics

The rates and molecular determinants of enzyme reactions profoundly dictate biological function. For example, the rapid responses of excitable cells, such as neurons and muscle cells, are dictated by the speed at which a substrate diffuses to a protein target[1, 2]. Many of these cellular responses result from the concerted, and evolutionarily optimized , activity of coupled enzyme-substrate and protein-protein association processes. These coupled reactions (such as in Fig. 1, panel A) can be further decomposed into individual binding events. As we will later discuss, Brownian dynamics simulations provide a powerful framework for probing such mechanisms, thus providing an essential bridge between descriptions of isolated binding events and their concerted reaction schemes to which they belong.

1.2 Single Binding Events

A single ligand/protein binding event is the minimal motif in cellular signal transduction pathways. For ease of discussion, we adopt a common perspective of dividing a single binding event into two phases [3, 4]: 1) The transient encounter, wherein two species approach from a distance to assume loosely bound complexes (Fig. 1B, green) and 2) The post-encounter, during which a subset of the transient encounter complexes settle into the final bound state (Fig. 1B, pink). The "transient encounter" is a nonlocal process governed by long-range diffusion and electrostatics, while the "post-encounter" is a local process dominated by short-range forces, conformational changes, and desolvation. A microscopic view of the interplay between these long-range and short-range interactions is essential to understand the binding mechanisms and their behavior in the cell cytoplasm [5]. From the standpoint of rapid reactions, this requires considering both the rates of formation of a transient encounter complex and the transition to the final bound state.

1.2.1 The transient encounter

The transient encounter describes the rapid, but generally intermediate assembly, of substrates with target enzymes [6, 7] as depicted in the top of Fig. 1B. This process begins with weak, long-range interactions accompanied by random molecular collisions. Early descriptions of such processes assumed a spherical target and a point substrate in solution, which could be solved analytically to yield an association rate [8]. Many biological binding events deviate from simple spherical models [9]. For instance, the diffusion of quinone within the mitochondrial membrane to shuttle electrons between respiratory complexes [10, 11] (Fig. 2A) would be amenable to a 2D diffusion model, for which analytically solvable models are possible [12]. Additional examples that deviate from the spherical paradigm but permit simple quantitative models include association of a tethered substrate with its target [13], such as the LIM domains within disordered proteins binding targets on muscle filaments [14](Fig. 2B), or the association of a particle undergoing free diffusion in 3D colliding with a surface [15], such as for an extracellular ligand activating a membrane-bound GPCR [16](Fig. 2C). One-dimensional diffusion-controlled binding events are also possible [17], such as the search a kinase might perform along an intrinsically-disordered protein containing a recognition motif for phosphorylation [18], or the binding events involving an ensemble of intermediate states [19]. Although simplified analytic or numerical models can be applied to these cases, atomistic detail or nontrivial interaction potentials often render infeasible approaches like these. Here, Brownian dynamics simulations have served as a complementary tool for capturing atomistic detail and nontrivial substrate/target interactions for predicting binding kinetics.

1.2.2 Post-encounter binding

The post-encounter event involves the transition of transiently bound states into their final, generally high-affinity (functional) poses (Fig. 1B). Intermediates from the transient-encounter complex have diverse poses that differ in terms of their propensity to form the bound-state. This process is driven by the free energy gains afforded through structural rearrangements that further enhance the chemical complementarity between the associated protein and ligand [20], as well as potential contributions from desolvating hydrated reactants and even quantum chemical effects like polarization and covalent binding [21]. Short-range forces governing association can impact the conformation ensemble by determining the geometric configurations and electrostatic interactions that permit guiding the system to its final, high affinity, bound-state. Furthermore, simulations that include explicitly-modeled water molecules show that the reorganization of solvent near binding interfaces can affect both the thermodynamics and kinetics of binding/unbinding [22–24]. The diverse mechanisms by which a transiently-bound substrate reached its post-encounter state can dramatically influence their respective binding rates [25, 26] Since the focus in this review is on forming the transient-encounter complex via diffusion, we refer the reader to robust discussions on this topic [25, 27–29]

1.3 Multiple Binding Events

1.3.1 Cooperativity, scaffolding, and signaling cascades.

Binding events in biological systems often feature complexities such as the involvement of multiple reactants, binding sites, or feedback mechanisms. Cooperativity and allostery (Fig. 2D-E) are two examples of these complexities. Cooperativity refers to when the binding of one substrate molecule facilitates or prevents the binding of a subsequent substrate of the same type [30]. A typical cooperative binding example is the binding between Ca^{2+} and calmodulin, a protein that has four Ca^{2+} binding sites called EF-hands [31] (Fig. 2D). Allostery more generally involves the binding of one molecule to a specific site that affects the binding of a different molecule to a distinct site [32]. Such allosteric regulation is often seen in the GPCR family, where agonists' binding to the cell-surface receptors induces heterotrimeric G protein binding on the intracellular side, a distance of nearly ≈ 40 Å away[33]. Another example includes substrate channeling (Fig. 2E), whereby intermediates are guided along enzyme-controlled pathways, often through spatial co-localization of enzymes [34]. Scaffolding (Fig. 2F) is another example of a complex binding event where molecules are brought together on a protein or other structure to facilitate a reaction or pathway, often through allosteric means. Here, Ca^{2+}-binding to TnC and its regulation of TnC-TnI interactions as part of the troponin scaffold [35] serves as a prominent example. Lastly, signaling cascades give rise to complex binding kinetics, such as the ATP-to-AMP-to-adenosine conversion involved in cancer immunosuppression [36] (Fig. 1A), or feedback-control, as seen in pathways involving molecules like Phospholipase C (PLC), Protein Kinase C (PKC), and Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Fig. 2G) [37].

1.4 Kinetic dysregulation in disease

The precise regulation of binding kinetics is not merely a matter of basic research; it is fundamental to health and disease. Altered enzyme kinetics can have detrimental effects on cellular homeostasis, hence a quantitative understanding of these processes in vivo and in vitro could give insights into human disease. For instance, dysfunctional kinetics in human developmental biology can disrupt the precise timing of cell division, maturation, and differentiation, leading to congenital defects and pediatric cancers [38, 39]. Similarly, a mismatch in reaction rates can be at the root of metabolic diseases. A classic example is Phenylketonuria (PKU), where mutations in the phenylalanine hydroxylase (PAH) enzyme lead to drastically reduced catalytic efficiency [40]. This kinetic defect prevents the normal conversion of phenylalanine to tyrosine, causing phenylalanine to accumulate and be converted into neurotoxic byproducts. In cardiac myofilaments, the precise timing of calcium binding is critical for muscle contraction. Certain mutations, such as the A8V mutation in cardiac troponin C (TnC), alter kinetics by increasing TnC's calcium sensitivity [41]. This enhanced binding impairs the heart's ability to relax, which is described as a form of diastolic dysfunction. Dysregulation of signaling cascades, a hallmark of cancer, is also profoundly influenced by kinetics. In neurological diseases, aberrations in the binding kinetics of proteins involved in neurotransmission can lead to the dysfunction of excitable cells [42]. Furthermore, drug design and delivery can be ineffective if drugs fail to occupy their targets for a sufficient duration, an effect governed by the drug's residence time [43].

1.5 The role of modeling with Brownian dynamics

Given the complexity and importance of these kinetic processes, computational modeling is an indispensable tool for synthesizing experimental data and generating novel hypotheses for testing. Our thesis in this review is that the Brownian dynamics (BD) technique is uniquely positioned for simulating complex, biomolecular processes and their kinetics. Therefore, in Sect. 2 we formalize the fundamental physical relationships underlying the binding scenarios outlined above.

2 The Molecular Physics of Binding Events

This section delves into the fundamental physical principles modeled by Brownian dynamics that govern single ligand-protein interactions. These principles provide a quantitative basis for understanding the binding process and its simulation. We also briefly introduce pertinent experimental methods to acquaint the reader with common techniques for evaluating ligand binding thermodynamics and kinetics. Computational approaches are discussed in Sect. 4.1.

2.1 Mass Action and Free Energy of Binding (ΔG)

We begin with the thermodynamic foundations that describe the equilibrium state of a binding reaction. The principles of mass action and free energy of binding (ΔG) are key for analyzing biomolecular interactions. The most basic relationship between substrates and products in a reversible reaction is:

$$A + B \underset{k_b}{\overset{k_f}{\rightleftharpoons}} A \cdot B \tag{1}$$

Here, B represents an apoenzyme that forms a holoenzyme complex (A·B) upon binding substrate A, with k_f and k_b representing the forward (association) and backward (dissociation) rate constants, respectively (see Fig. 3A). At equilibrium, the dissociation constant (K_D) is defined as:

$$K_D \equiv k_b/k_f \tag{2}$$

$$K_D = \frac{[A][B]}{[A \cdot B]} \tag{3}$$

$$\Rightarrow \quad \ln K_D = -\frac{\Delta G}{k_B T} \tag{4}$$

The last line reflects the relationship between the standard Gibbs free energy of binding (ΔG) and the dissociation constant.

We next outline the physical factors contributing to ΔG. The binding affinity (K_D) indicates the equilibrium distribution between the bound and unbound states of a molecule. K_D values for biomolecules generally range from picomolar (pM) to millimolar (mM). While ΔG indicates the spontaneity of a reaction, the k_f and k_b rates determine its feasibility on biological timescales (e.g., whether the system is in rapid equilibrium or a non-equilibrium state). As a state function, ΔG depends only on the initial and final states, revealing the net change in free energy but not the specific reaction pathway. From a molecular perspective, this thermodynamic quantity arises from the sum of all energetic interactions among the reactants, products, and the surrounding solvent, often as a partition function [44]. The change in free energy upon binding, $\Delta G = \Delta H - T\Delta S$, can be decomposed into its enthalpic (ΔH) and entropic (ΔS) components; the balance between them is a critical determinant of the interaction. The partition function reflects interactions not only between the binding partners (e.g., enzyme and substrate) but also with the solvent.[45]. The solvent also plays a crucial role, particularly through the hydrophobic effect, which drives the assembly of nonpolar surfaces to minimize their energetically unfavorable contact with water. The release of water bound to the reactants or the target binding site also typically contributes to substantial entropy gains upon binding [46]. The complexity of these interactions motivates the use of simulation techniques like Molecular Dynamics (MD) simulations to extensively sample the ensemble of thermodynamically accessible states and their associated energies. It is also important to note that Eq. 1 also highlights the importance of local concentrations of the ligand [A] and protein [B] that may deviate from their bulk values, which can drive product formation. Such local concentrations arise in contexts such as substrate channeling or tethering.

2.2 Association and Dissociation

While thermodynamic quantities like K_D are based on a system at equilibrium, rate constants help determine how quickly the system reaches equilibrium. For a system that is out of equilibrium, it

is practical to follow the time-dependent concentrations changes for reactants, which for a system like Eq. 1, a reaction scheme is used

$$\frac{d[A]}{dt} = -k_f[A][B] + k_b[A \cdot B] \tag{5}$$

where k_b represents the dissociation rate, the forward rate constant, k_f, often represents a multi-step process. In a system consisting of diffusible substrates, the forward rate is commonly decomposed into an initial rate of transient encounter ($\mathrm{k_{on}}$) and a rate for forming the final complex (k_{int}), The overall forward rate constant (k_f) incorporates the rates for both the diffusional encounter ($\mathrm{k_{on}}$) and any subsequent intrinsic step (k_{int}), as given by [47, 48]:

$$k_f = \frac{k_{on}\, k_{\mathrm{int}}}{k_{on} + k_{\mathrm{int}}} \tag{6}$$

where $\mathrm{k_{on}}$ represents the diffusive encounter rate and k_{int} the intrinsic reaction rate.

The overall rate of a reaction is often dictated by its slowest step, leading to two distinct kinetic regimes. A reaction is reaction-limited when the intrinsic chemical or conformational step is much slower than the diffusional encounter rate ($k_{on} >> k_{int}$), causing the overall rate to be governed by the intrinsic rate ($k_f \rightarrow k_{int}$) per Eq. 6. Conversely, a reaction is diffusion-limited when the intrinsic rate is much faster than the association rate ($k_{int} >> k_{on}$), and the overall rate becomes dependent on the rate of diffusional encounter ($k_f \rightarrow k_{on}$). A formal definition is discussed in detail elsewhere [48].

2.2.1 Factors governing $\mathrm{k_{on}}$: Diffusion-Limited

Many biological reactions are limited not by the chemical step, but by the rate of diffusional search. For these cases, $\mathrm{k_{on}}$ reflects the solute's traversal of a potential energy landscape to form an encounter complex (see Fig. 3). These rates typically range from 10^5 to 10^9 $\mathrm{M^{-1}s^{-1}}$ in the diffusion-limited regime [49], compared to intrinsic catalytic rates (k_{int}) that often vary from milliseconds to seconds [50].

Physical factors across a hierarchy of spatial scales, from long-range diffusion to local interactions, collectively shape substrate/target association. The diffusion coefficient (D) is a primary factor at large distances and is derived from the friction of random solvent collisions (Brownian motion) and scaling with the Stokes radius. However, hydrodynamic interactions arising from the displacement of solvent by the approaching solute can reduce the protein-protein $\mathrm{k_{on}}$ relative to cases where these interactions are negligible [51].. Additionally, the dimensionality of the problem domain determines collision timescales, since searches constrained to 1D or 2D often proceed more efficiently than those in bulk 3D .

In parallel, long-range mean-field electrostatic interactions can steer molecules toward one another, often increasing rates by orders of magnitude [49]. The barnase-barstar interaction [52] is a classic example, where electrostatic steering drives binding rates to 10^8–10^9 $\mathrm{M^{-1}s^{-1}}$ [52]. Similar electrostatic acceleration is observed in intrinsically disordered proteins, such as the CaMBR region of calcineurin binding to calmodulin, even where conformational gating governs the final locking step [53]. Following this initial approach, the system transitions toward a more tightly associated state where short-range forces become dominant (Fig. 4, bottom)

2.2.2 Factors governing $\mathrm{k_{on}}$: Reaction-Limited and post-encounter effects

Reaction-limited systems typically involve the chemical conversion of a substrate into a product. However, post-encounter effects can also contribute to reaction-limited timescales. Post-encounter effects encompass the events that occur between the transient encounter of a substrate with its target and the substrate's eventual accommodation into the binding site.Eq. 6 quantifies how post-encounter processes influence k_{int} in the expression for the forward rate. The energetic cost associated with the conformational adjustments the protein and its substrate must undergo to achieve the final bound state is an important consideration connected with post-encounter events.Some of these contributions stem from local interactions that provide enthalpic stabilization. The desolvation energy can also be an important factor, which is the energetic cost of removing water molecules from the interacting surfaces of the binding domain. This process is closely linked to dewetting phenomena, in which transient transitions between wet and dry interfaces influence binding kinetics [54], as well as to "dangling" hydrogen bonds at hydrophobic solute–water interfaces [55] , which can lubricate conformational changes during binding. Entropy may also increase as hydrating waters are released from the active site, gaining configurational freedom that can offset the entropic losses associated with confining both ligand and target to their bound conformations.

2.2.3 Factors governing k_{off}

While the association rate often governs the initial binding event, the dissociation rate, or k_{off}, determines the duration of the interaction and is often a more critical parameter for biological function.[43]. In fact, in vivo drug efficacy often correlates more strongly with the dissociation rate (k_{off}) [56]. For a binding event without a subsequent chemical reaction, the interplay between the association (k_{on}) and dissociation (k_{off}) rates determines both the kinetics and the thermodynamic equilibrium of the system. For ligands whose binding kinetics are diffusion-limited, differences in binding free energies are largely dictated by the intrinsic dissociation kinetics of each ligand. Fundamentally, the rate of dissociation (k_{off}) reflects the stability of the bound complex and is governed by the dominant energy barrier separating the bound and unbound states. That barrier's height is shaped by physical factors such as the need for the ligand to break strong short-range non-bonded interactions and the entropic cost of solvent reorganization as it is resolved. Both hydrophobic and hydrophilic ligands tend to impose order on surrounding waters and thereby reduce entropy. Additional entropic contributions arise from the protein's rehydration and changes in its conformational rearrangements, along with enthalpic penalties as native contacts are reformed.

3 Experimental approaches for characterizing thermodynamics and kinetics of binding

3.1 Assays for slow reactions or steady state conditions

To experimentally measure these thermodynamic values and validate theoretical models, several biophysical techniques can be employed. While a comprehensive review of kinetic assays is beyond our scope , we will highlight several key methods.

Isothermal Titration Calorimetry (ITC) Among the core experimental techniques used to quantify binding thermodynamics like $\Delta G, \Delta H$, isothermal titration calorimetry (ITC) is the gold standard. [57]. ITC directly measures heat released or absorbed upon binding [57], through incremental additions of ligand. Hence, ITC is an ideal tool for capturing the thermodynamics of binding resulting from formation of the post-encounter state. ITC can also be applied to allosteric and cooperative binding events to help decipher the underlying mechanism. However, in these complex cases, fitting the ITC data requires extra caution, as different binding models can produce equally good fits to the same dataset [58]. For such systems, the variable-c ITC strategy, performing repeated titrations of ligand into a range of protein concentrations can be necessary. This was, for example, carried out by Bonin et al. [59] to quantify cooperative binding of dUMP to thymidylate synthase (TS).

In addition to ITC, fluorescence-based techniques discussed later in Sect. 3.2.2 can also be used to infer cooperative and allosteric binding mechanisms by monitoring titration curves [60, 61]. For instance, by utilizing the intrinsic tryptophan fluorescence of the glucokinase regulatory protein (GKRP), Zelent et al. revealed the cooperative interactions of various allosteric ligands on the binding of glucokinase to GKRP [62].

Classical experimental techniques, such as colorimetric assays and Western blotting, are often well suited for measuring slow reaction-limited processes. Colorimetric assays monitor color changes as the reaction progresses and remain a foundational method for high-throughput screening [63]. Early quantitative measurements of biochemical reactions often focused on enzyme kinetics [64]. Much of this work was performed using spectrophotometers, which measure reaction rates by monitoring changes in absorbance. These methods are particularly well-suited for capturing reaction-limited processes, as they are not fast enough to measure rapid diffusion-limited processes [64]. In contrast, Western blotting is a technique used to identify specific proteins and was not originally designed for measuring kinetics. Western blots have been adapted for kinetic studies by performing a series of time-dependent assays [65], although they are often labor-intensive and have low throughput. Additionally, kinetic ITC can determine the kinetic rate constant for enzyme-inhibitor interactions by monitoring steady-state heat production from an enzyme reaction [66], but is generally not particularly rapid.

3.2 Techniques for Rapid Kinetics and Conformational Dynamics

3.2.1 Fluid-Phase and Surface-Based Kinetics

Stopped-flow for rapid kinetics Measuring rapid, diffusion-limited rates requires specialized experimental techniques capable of capturing events on the millisecond timescale. Stopped-flow spectroscopy is a powerful tool for studying rapid biomolecular kinetics, with a key advantage being its short dead time before reliable measurements can be made is on the order of milliseconds.

This rapid dead time makes it suitable for measuring fast k_{on}values [67], and it is well-suited for measuring very fast reaction rates [68]. One of the earliest stopped-flow apparatuses was developed by Chance and Gibson [69]. Since then, it has been used in a variety of applications, including capturing fast pre-steady-state kinetics and resolving transient intermediates [70]. In addition, Surface Plasmon Resonance (SPR) is often used for kinetics (k_{on}, k_{off}), including those influenced by diffusion [71].

3.2.2 Structural Dynamics

Fluorescence-based methods Single-molecule fluorescence labeling methods are widely used to study the conformational dynamics of biomolecules [60] and have proven to be useful tools for investigating binding properties, including kinetics and affinities [61]. The basic principle involves labeling binding partners with fluorescent dyes; by monitoring signal changes that reflect the spatiotemporal distribution of these partners, information on conformational dynamics and binding rates can be extracted. Among these approaches, single-molecule fluorescence resonance energy transfer (smFRET) is particularly well suited for probing biomolecular binding events because its signal is highly sensitive to the distance between the two labeled partners. smFRET relies on non-radiative energy transfer from a donor dye to an acceptor dye. When these dyes are attached to separate binding partners, the technique becomes extremely sensitive and precise for characterizing binding events [61]. smFRET-based kinetic measurements have been successfully applied, for example, to study complexin binding to the SNARE complex [72]. Owing to its single-molecule resolution, smFRET can also reveal conformational changes that occur upon association [73].

Fluorescent probe labeling techniques are also commonly used to measure the diffusion behavior of biomolecules in heterogeneous environments, with Green Fluorescent Protein (GFP) being a widely used tag for proteins of interest. For example, labeling the G3BP1 protein with GFP enabled monitoring of its liquid–liquid phase separation (LLPS) in cells and characterization of its diffusion behavior both before phase separation and within the resulting condensates [74]. This GFP-labeling strategy for studying protein dynamics within condensates has been broadly applied, including in bacterial cells [75] and in nuclei where transcription factors form condensates [76]. For smaller molecules, fluorescent dyes are often used to label metabolites such as ATP or other signaling compounds to characterize their diffusion in cellular environments. For instance, by labeling ATP with a fluorescent dye, Vendelin and colleagues showed that ATP diffusion is hindered by muscle filaments in rat cardiomyocytes [77]. Even fluorescent dyes alone can be loaded into cells to measure overall cargo-transport dynamics in the cytoplasm. A representative example is again found in the G3BP1 LLPS study, where quantum dots were co-loaded with GFP-labeled G3BP1, enabling simultaneous characterization of crowder diffusion (via the quantum dots) and LLPS formation dynamics (via the GFP label) [74].

Nuclear Magnetic Resonance (NMR) Spectroscopy As a complementary approach, NMR spectroscopy provides a unique window into atomic-level details of kinetic processes across a broad range of timescales. NMR explicitly probes motion and exchange processes spanning picoseconds to seconds [78]. With this capability, NMR yields information on molecular structure, dynamics, and kinetics at atomic resolution [79]. Bond rotations on the ps–ns scale can be estimated via relaxation measurements. Relaxation dispersion techniques characterize conformational exchange on the μs–ms timescale [78], while two-dimensional Exchange Spectroscopy (2D EXSY) experiments capture slower exchange rates, such as dissociation processes occurring on the order of seconds [78]. Altogether, NMR is a powerful tool for dissecting complex kinetic mechanisms, including conformational gating [78], whereby access to a binding site is dynamically modulated.

Experimental methods that capture protein structural changes upon perturbation can provide a structural basis for cooperative binding. Solution NMR spectroscopy, in particular, offers dynamic structural insight. For example, solution NMR data for the KIX domain in a binary complex with c-Myb and in a ternary complex with c-Myb and MLL were used to decipher the structural basis of positive cooperative binding of these ligands to KIX [80].

NMR spectroscopy can also be used to measure binding rates by tracking changes in residue-specific relaxation rates before and after ligand binding [81]. A key advantage of NMR-based methods is their ability to characterize weak binding events, which typically exhibit fast dissociation rates [81]. Additionally, because both smFRET and NMR can resolve protein conformational states and monitor their changes upon ligand binding, these methods are well suited for deciphering binding mechanisms—distinguishing, for instance, between induced-fit and conformational-selection pathways [82, 83].

3.3 Methodologies for Dissociation Rate Measurement

3.3.1 Overview of methods

SPR, Fluorescent labeling, and Radiolabeling are the top three methods. Measuring protein-ligand unbinding rates [84] is critical for understanding drug efficacy (residence time). Several experimental techniques are particularly well-suited for measuring dissociation rates. SPR, Fluorescent labeling, and Radiolabeling are the top three methods for measuring protein-ligand unbinding rates [84], which we describe below.

3.3.2 Label-Based Approaches

The discovery of Green Fluorescent Protein (GFP) greatly advanced cell biology, enabling the observation of molecular processes directly within living cells [85]. This discovery paved the way for techniques that could visualize not just the location of proteins but also their interactions in real time. To observe these kinetic events within their native cellular environment, methods based on fluorescent labeling are commonly used. In Förster Resonance Energy Transfer (FRET) as an example, fluorescent proteins provide a mechanism to measure time-dependent changes in the distances between interacting probe molecules [86].

Live-cell imaging with FRET provides dynamic, real-time information on interaction kinetics and is enabled by tagging proteins with a FRET pair. Introduction of fluorescent probes is typically done by genetically tagging interacting proteins with a pair of fluorescent probes (a donor and an acceptor) to monitor their association over time [87]. Overall, live-cell imaging with FRET allows for the real-time study of protein interactions in the 1-10 nm range [86]. As a result, FRET can provide dynamic, often quantitative, information on interaction kinetics [86] and associated conformational changes. FRET-based sensors can be instrumental for monitoring in vivo signaling events, from changes in conformation owing to PTMs or substrate binding, or co-association of components forming functional complexes.

FRET can also be adapted to measure drug-target binding kinetics, typically through competitive assays, where unlabeled drugs compete against a fluorescent probe for binding to the target, yielding kinetic and thermodynamic data [88]. These high-throughput kinetic probe competition assays have been used to survey drug kinetics across broad classes of protein kinases [89].

There are additional advancements that provide further precision, including single-molecule FRET and fluorescence lifetime imaging. These enable the study of conformational dynamics and binding events at the single-molecule level. As a further example, radiolabeled drugs also use competition assays to measure kinetic parameters, such as for drug-GPCR binding kinetics [90], but are generally not suitable diffusion-limited reactions.

3.3.3 Label-Free Real-Time Monitoring

While labeling methods are powerful, they perturb the system and can introduce non-physiological effects; thus, label-free techniques provide an important alternative [91]. Label-free techniques like SPR and BLI are widely used for real-time study of biomolecular interactions that can be used to extract kinetic parameters. These methods immobilize one binding partner on a sensor surface and flow the other partner over it, monitoring binding events without the need for fluorescent or radioactive labels. SPR detects a refractive index change at a metal-film interface [92]; in the dissociation phase where the bound ligand dissociates from the immobilized chip, the decayed SPR signal is indicative of dissociation rates [93].In contrast, BLI measures the interference pattern of light reflected from two surfaces [94], with the shift being proportional to the number of bound molecules. BLI is less sensitive to bulk refractive index changes compared to SPR [94]. The resulting data from SPR or BLI are plotted to give the binding response over time, which can highlight the association, saturation, and dissociation phases of the interaction [94]. Kinetic parameters such as k_{on}, k_{off}, and the equilibrium dissociation constant (K_D) are extracted by fitting kinetic models to these phases.

3.3.4 Structure determination

Although not a requisite for conducting kinetic and thermodynamic assays, structural data can be instrumental in interpreting assay data and devising hypotheses. Namely, atomistic resolution structures of enzymes can give important information about their function. Classically, X-ray crystallography has been a standard for interpreting binding data, such as for revealing cooperative binding between transcription factors and DNA [95]. More recently, NMR techniques and cryo-EM have emerged as strong complements to x-ray. Ab initio structure prediction is also becoming increasing popular using techniques such as AlphaFold and RoseTTAFold [96, 97]. A survey of experimental and ab initio structure determination methods provide ample overview of the state of the art in this area [98, 99].

4 Modeling diffusion-limited kinetics with Brownian dynamics

MD and BD are the most common simulation approaches for modeling substrate/target binding, using detailed molecular structures of the proteins. Briefly, MD provides all-atom detail but is limited in simulating diffusional encounters, while continuum models lack explicit molecular details. The efficiency of BD stems from its implicit treatment of the solvent versus all-atom simulations that use explicit solvent. BD makes practical the simulation of thousands of trajectories (Fig. 1B), which enable its use for studying kinetic processes. BD provides a flexible framework for accounting for a variety of physicochemical factors that influence interactions within and between biomolecules, from solvent contributions to electrostatic potentials. Beyond kinetics, its utility extends into equilibrium properties as well, such as for the prediction of flexible systems like intrinsically-disordered proteins. We posit that Brownian dynamics is an ideal framework for balancing the detailed resolution of molecular dynamics (MD) with the computational efficiency of continuum models to efficiently model the concepts highlighted above. To demonstrate BD's utility, it is helpful to establish its fundamental basis, then compare it to other simulation techniques used for probing transient encounters and coupled binding events. Therefore, in this section we discuss a variety of paradigms in the modeling of kinetics, including the physical bases and where BD fits in as a modeling tool.

4.1 Fundamentals of the Brownian method

The mathematical foundation of BD lies in the Langevin equation, which describes stochastic motion of a solute, subject to friction and an external potential:

$$m\frac{d\mathbf{v}}{dt} = -\gamma\mathbf{v} + \mathbf{F}(\mathbf{r}) + \sigma\mathbf{N}(t) \tag{7}$$

Here, the inertial term ($m\frac{d\mathbf{v}}{dt}$) is balanced by the frictional drag arising from the solute's interaction with the solvent ($-\gamma\mathbf{v}$), forces from molecular interactions ($\mathbf{F}(\mathbf{r})$), and a random force (amplitude σ) from solvent collisions ($\mathbf{N}(t)$). The force term, $F(r)$, captures the effects of long-range interactions as well as local van der Waals encounters, and can be evaluated either explicitly as pairwise interactions or implicitly by a potential of mean force (PMF). The PMF is an effective free energy landscape that averages degrees of freedom orthogonal to the reaction coordinate, including the solvent degrees of freedom [44]. The friction term represents the solvent's contribution to the solute's mobility and can include hydrodynamic effects.

The fundamental assumption of BD is that for a particle in a viscous solvent, the system is in the high-friction limit, where the frictional drag term dominates the inertial term. This allows the inertial term to be considered negligible ($m\frac{d\mathbf{v}}{dt} \approx 0$), which gives rise to the overdamped Langevin equation and is a simplification that is at the heart of BD calculations :

$$\mathbf{v} = (D/k_BT)\ \mathbf{F}(\mathbf{r}) + \sqrt{2D}\ \mathbf{N}(t) \tag{8}$$

where we have used the Einstein relation:

$$D = (k_BT)/\gamma \tag{9}$$

and $D = \sigma^2/(2\gamma^2)$ to express the equation in terms of the diffusion coefficient. The friction (diffusion) coefficient arises fundamentally from the fluctuation-dissipation theorem (Eq. 10), which relates the random bumps the solvent makes on the solute to the frictional drag experienced by the solute. More generally, BD handles solvation effects by relating the diffusion coefficient to dissipation, which is influenced by factors like solvent viscosity.

In aqueous media, force fluctuations arising from the random movements of water molecules transfer momentum to their surroundings. This results in the dissipation of energy from the system through friction. Together, these contributions are closely tied to the diffusion coefficient through the fluctuation/dissipation theorem. To demonstrate this, we refer to a Green-Kubo relation [100] that provides the friction coefficient from the integral of the random force autocorrelation over time

$$\gamma = \frac{1}{3k_BT}\int_0^\infty \langle \mathbf{F}_{rand}(t)\cdot\mathbf{F}_{rand}(0)\rangle dt \tag{10}$$

where $\mathbf{F}_{rand}(t)$ corresponds to the momentary random force, which is reflected as $\sigma\mathbf{N}(t)$ in Eq. 7 [100]. The diffusion coefficient, D, is then determined by the Einstein-Smoluchowski relation (Eq. 9) using this estimate of the friction coefficient. Later in Sect. 4.2 we will show how k_{on} is positively correlated with increasing D.

These concepts readily extend to other solvents, including composite environments such as oil-water interfaces or heterolipid membranes. Beyond dissipation through random solvent collisions, the translational and rotational diffusion of larger solutes can present significant intramolecu-

lar hydrodynamic interactions. To characterize these, oftentimes autocorrelation functions of a solute's position or velocity are used in lieu of Eq. 10 [101, 102]:

$$D = \frac{1}{3N} \sum_{i=1}^{N} \int_0^\infty \langle v_i(t')\, v_i(0) \rangle \, dt' \tag{11}$$

where Ermak and McCammon provided an extended form of Eq. 8 to capture translational and rotational degrees of freedom, inclusive of hydrodynamic interactions [103], using coefficients following Rotne-Prager [104].

4.2 Transient encounter: BD for Homogeneous Media

4.2.1 Modeling Rigid Solutes

Early treatments of enzyme kinetics involved model (oftentimes spherical) systems, for which rates were predominantly determined by a substrate's diffusion to a reactive site. We overview some of the important kinetic frameworks arising from this simple system. We begin with the Smoluchowski model describing diffusion toward a spherical enzyme. From there, we discuss Kramers' theory to address the concept of an energy barrier that was neglected in the early Smoluchowski descriptions. We next present more modern and rigorous ways of addressing reaction kinetics, including committor analysis, Markov models, and Survival Probability as practical tools for analyzing molecular simulation data and estimating kinetic information.

Kinetic models

The Spherical Model The study of diffusion-limited reactions often begins with the simplest possible case: a spherical target. While a clear simplification, this model serves as a powerful and useful approximation that provides a baseline for understanding diffusion-limited reactions. It acts as a starting point from which refinements that account for more realistic protein shapes, electrostatic interactions, and reactive surface patches can be developed. For instance, the Debye-Smoluchowski model[105] describes a particle diffusing and reacting with a spherical enzyme that has a centrosymmetric interaction potential. This is usually framed (in steady state) as a Laplace problem of the form

$$\nabla \cdot (D(R) \nabla c(R)) = 0 \tag{12}$$

$$s.t.\ c(R) = 1 \quad \text{on } \Gamma_{outer} \tag{13}$$

$$c(R) = 0 \quad \text{on } \Gamma_{enzyme} \tag{14}$$

These equations describe the relationship between the concentration $c(R)$ and the diffusion coefficient $D(R)$ at a given position R. The system is constrained by two boundaries: Γ_{outer} (the outer boundary) where $c(R) = 1$, and Γ_{enzyme} (the enzyme surface) where $c(R) = 0$. This can be obtained by analytic or numerical solutions of a reaction-diffusion equation, given suitable boundary conditions:

$$k_{on,0} = 4\pi R D \tag{15}$$

When a potential of mean force is important to the association event, the Smoluchowski equation is often used

$$\frac{\partial p(\mathbf{r}, t)}{\partial t} = \nabla \cdot D \left[\nabla p(\mathbf{r}, t) + \beta p(\mathbf{r}, t) \nabla U(\mathbf{r}) \right], \tag{16}$$

where we are now representing the probability density of particles by p, D is the diffusion coefficient, $\beta \equiv (k_B T)^{-1}$ is the inverse thermal energy, $U(\mathbf{r})$ is the is a drift term from the interaction potential of mean force, $p(\mathbf{r}, t)$ normalized: $\int p(\mathbf{r}, t)\, d\mathbf{r} = 1$. Although it is beyond the scope of this review, it can be shown that an ensemble of particles undergoing Brownian motion given $F(r) = -\nabla U(r)$ and under certain assumptions can be expressed as a continuum via Eq. 16 .

Electrostatic interactions are typically the basis of PMFs far from the transient encounter complex as they decay slowly over distance. Here, the Poisson-Boltzmann equation, which relates charge distribution, ionic strength, and boundary conditions (such as molecular surfaces) to the electric potential, is commonly used [106]:

$$\nabla \cdot (\epsilon(\mathbf{r}) \nabla \phi(\mathbf{r})) = -\rho(\mathbf{r}) - \sum_i z_i e n_i^\infty \exp\left(-\frac{z_i e \phi(\mathbf{r})}{k_B T} \right) \tag{17}$$

Here, the term within the summation reflects the bulk solution's ionic strength and how its distribution deviates near the charged surface. The resulting potential is multiplied by a substrate's charge to yield the electrostatic energy, $U = q\phi$. This equation is usually solved with tools like

DelPhi [107] and APBS[108]. To quantify their effect on k_{on}, the Debye model can be used [109], which adds an additional term to the Smoluchowski rate to account for long-range electrostatic interactions. Then, with a long-range interaction potential, we can use [105, 110]

$$k_{on} = \frac{4\pi D}{\int_{r=R}^{r=\infty} e^{\beta U(r)} r^{-2} dr} \tag{18}$$

More generally, k_{on} can be determined from the total flux to a reactive surface [109]

$$k_{on} = -\frac{1}{p_{bulk}} \int_{\Gamma_r} \hat{n}(x) \cdot D(x)[\nabla p(x) + \beta p(x) \nabla U(x)] d\Gamma \tag{19}$$

where Γ_r is the reactive region on the enzyme surface (Γ), $\hat{n}(x)$ is the surface normal along Γ, $x \in \Gamma$ and $p(x)$ is the steady-state solution to Eq. 16. We note that the interaction potential can have significant impacts on both k_{on} and k_{off}. A favorable gradient of the potential that accelerates the association rate can result in a deeper energy well that reduces the escape rate for bound substrates, thereby slowing the k_{off}. These forms can be solved exactly or approximately for only idealized geometries, which limit their real-world application to arbitrary substrate/target binding events.

Brownian dynamics Brownian Dynamics is arguably the first mature computational method well-known for streamlining the calculation of the kinetics for diffusion limited association (k_{on}), of two, atomistically-detailed proteins (A and B in Eq. 1) [4, 111, 112]. To achieve this, its simulations rely on coarse-grained representations of the reactants to model their diffusional approach from a separated state to form a transition or encounter-complex ensemble [111]. The rate scales with the number of successful collisions. BD simulations are typically associated with time scales spanning nanoseconds to microseconds. This is precisely the regime for monitoring fast diffusive processes and binding events that are difficult to capture with atomistic simulations. In these simulations, the interaction forces between molecules can be defined arbitrarily, though they are often derived from an electrostatic potential calculated by solving the Poisson-Boltzmann equation [112].

The Northrup-Allison-McCammon (NAM) algorithm [4] is among the most well-known tools for computing association rates from BD simulations (Fig. 5). These simulations typically start from experimentally determined structures (e.g., X-ray crystallography, NMR spectroscopy, or cryo-EM) or from predicted models generated by docking, homology modeling, or ab initio methods. This approach efficiently bridges short-range BD simulations with analytical long-range diffusion, enabling practical calculation of association rates. The simulations follow Langevin-type equations of motion, where the force term is often derived from a numerically estimated electrostatic potential. This versatile method applies to both encounter complex and functional complex formation, provided specific reaction criteria are supplied. The NAM simulations begin by initiating numerous trajectories, starting with one molecule placed randomly on a "b-sphere" of radius b around a second, fixed molecule. Each diffusive trajectory is simulated until the molecules either separate to an outer "c-sphere" or meet the predefined binding criteria. The overall rate is determined from key parameters, including β, the fraction of trajectories that successfully reach the bound state. Another key parameter is $k(R)$, the diffusion rate at separation R, which within the b-c layer is described by the Smoluchowski expression in Eq. 15.

An early example from Northrup *et al*[113] demonstrated through BD simulations that, for protein-protein association involving reactive patches, water molecules in the encounter complex play a stabilizing role. This stabilization accelerates the formation of the functional complex by allowing more opportunities for re-collision between the proteins [113]. This solvent effect explains the fast measured association rate constants k_{on} of 0.5–5 $\times$ 10^6 $M^{-1}s^{-1}$ for protein-protein associations with specific geometric requirements [113]. Similarly, Gabdoulline *et al* used BD to predict the association rates for five globular protein-protein pairs with varying degrees of intermolecular electrostatic steering, showing that BD can accurately predict k_{on} values that align with experimental measurements [114]. More recent BD packages commonly reported in the literature include UHBD[115], SDA7[116], Browndye[111], BD-BOX[117], ReaDDy2[118], GeomBD3[119], as well as hybrids that combine BD and MD (e.g., SEEKR[120]). The differences between these packages stem from their specific target applications, the level of approximation they use, and the features they prioritize. For example, Browndye is specialized for predicting the electrostatic-steered binding rates, ReaDDy2 is designed for reaction-diffusion networks etc. An excellent review summarizing these BD-based packages is given by [27].

While BD is an improvement on MD, it can still be subject to significant computational expense, especially for large or highly-flexible systems. To address the computational cost, variants of the NAM algorithm, such as weighted-ensemble BD [121] and biased-BD [122], have been developed. These methods accelerate the sampling of binding events, either by preferentially exploring trajectories that move toward the bound state or by applying a biasing force to speed

up the process.

Although the NAM algorithm and BD as a whole are predominantly used for computing association rates, there are examples of BD being applied for k_{off}. These cases are typically applicable to events where dissociation occurs through a 'barrier-less' diffusive escape. An example of which includes using BD to simulate microtubule disaggregation [123]. However, many biological systems have non-trivial and sometimes multiple transient encounter states, or significant barriers bridging the transient and post-encounter states. To address more general kinetic modeling of biological systems, we now introduce a few frameworks for estimating kinetic rates that complement or extend BD simulation approaches.

Kramers theory We first discuss Kramers theory and Transition State Theory (TST), which can be used to estimate the rate of escape of a bound particle over a potential energy barrier (see Fig. 3). This approach is appropriate for binding events that are reaction-limited For TST due to Henry Eyring, a bound particle is assumed to be equilibrated in a potential well representing the bound state with a curvature of ω_a and confined by a barrier of amplitude $\Delta G^{\ddagger}$ [124] (seeFig. 3A). Hendrik Kramers extended this theory by assuming that the particle undergoes Brownian motion with viscous drag, and that the barrier assumes a simple parabolic potential of mean force. Under these assumptions, it was shown that

$$k_{Kramers} = \kappa_K \left[\frac{\omega_A}{2\pi} \exp\left(-\frac{\Delta G^{\ddagger}}{k_B T} \right) \right] \tag{20}$$

$$= \kappa_K k_{TST} \tag{21}$$

where the short-form of Kramers transmission coefficient κ represents the influence of friction on the observed escape rate [125] In the high-friction limit most appropriate for biological systems, $\kappa \to \omega_b/\gamma$, where ω_b reflects the particle's oscillations at the barrier. As the friction γ increases, $k_{Kramers}$ is reduced relative to k_{TST}. The low-friction limit yields $\kappa \to 1$, upon which $k_{Kramers} \to k_{TST}$ is recovered. While originally formulated for escape (dissociation) processes, it can also be extended to association phenomena when an effective free-energy barrier governs the encounter. A comprehensive review of this theory is available for an elaboration of these concepts [125].

The Kramers approach differs from the Brownian dynamics framework as the potential energy surface (PES) governing the diffusive encounter is replaced with a single barrier between bound and unbound states. It can, however, be used in conjunction with BD if the most significant barrier along the reaction coordinate sits between the diffusional encounter and the bound state. Such an assumption was used for BD simulations involving buried binding sites, whose barrier to entry could be described with a one-dimensional potential of mean force [126]. Including a local diffusion coefficient along the PMF can further improve the accuracy of the kinetic predictions [127].

Committor probabilities The reaction path in Fig. 3A is generally a simplification of the binding process that is amenable to treatments like Kramer's theory. A more representative ligand/protein association landscape for biomolecules, is represented in Fig. 3B, which includes a yellow reaction coordinating linking the transitional encounter and bound state. This path traverses a variety of saddle points that represent barriers along the association path, which would challenge the application of models like Kramers' theory.

Instead, Committor probabilities provide a more general mechanism to identify transition state barriers along a reaction pathway, and to accommodate more complicated free energy barriers than the parabolic PMFs assumed in Kramers' models and can include surfaces. Importantly, it provides a framework for the numerical estimation of reaction barriers that one obtained from molecular simulations. Rather than relying on a predefined reaction coordinate, the committor probability ($p_B(\mathbf{x})$) quantifies the likelihood that a trajectory initiated from a configuration ($\mathbf{x}$) will reach the product state B before returning to the reactant state A .

$$q(x) = \text{Prob(Trajectory from 'x' reaches B before A)} \tag{22}$$

, where here we use $q(A)$ and $q(B)$ to signify the unbound and bound states, respectively. The transition-state surface is then defined by configurations where the committor equals one half ($q(x) = 0.5$), meaning the system is equally likely to commit to either state. To arrive at rate constants, the equilibrium probability density ($\rho(x)$) and the probability flux, ($D(x)\nabla q(x)$) are evaluated along the 'isocommittor' surface where $q = 0.5$. Integration along the isocommittor surface and normalization by the total probability of being in A yields the rate constant for transitions from state A to B:

$$k_{A\to B} = \frac{\int_{\Gamma_{iso}} \rho(x) D(x) \nabla q(x) \cdot \Gamma_{iso}}{\int_A \rho(x) dx} \tag{23}$$

. where Γ_{iso} is the isosurface where $q = 0.5$ Implied in this model is a form of detailed balance for the reactive fluxes, which assert that the net probability flux between states vanishes at equilibrium

$$k_{A\to B}\pi_A = k_{B\to A}\pi_B \tag{24}$$

where (π_A) and (π_B) are the equilibrium populations of states A and B, and $(k_{A\to B})$ and $(k_{B\to A})$ are the corresponding transition rates. The committor framework forms the theoretical basis for trajectory-based methods such as milestoning [128].

Markovian Framework Markov state models (MSMs) provide an alternative approach for predicting binding kinetics, which relies on a pre-defined set of microstates representing fully-bound, fully-dissociated, and intermediate states. These strategies rely on the discretization of the state space into regions that permit transitions from one state to adjacent states, independent of their origin, thereby linking long-lived and functionally important conformational states of proteins. ideally, the discretization provides a suitable representation of a reaction pathway and ideally one that encompasses biologically relevant states—such as catalytic, fully-dissociated, and bound conformations.

The Markov state modeling approach seeks to define a transition probability matrix, $\mathbf{T}(\tau)$, that can evolve a given microstate population (such as at time t for $P(t)$,) by τ to obtain their probabilities, P$(t+\tau$):

$$P(t+\tau) = P(t)\mathbf{T}(\tau) \tag{25}$$

The resulting system satisfies conditions such as detailed balance between states P_i and P_j:

$$P_i T_{ij} = P_j T_{ji} \tag{26}$$

and that transitions from a given t to $t+\tau$ are independent of the preceding steps.

Given a Markovian model that encompasses functionally-relevant states of a system, steady-state and time-dependent probabilities across the microstates can be readily determined. Relaxation rates for the microstates implied at lag time τ can also be estimated from the eigenvalues, λ of $\mathbf{T}(\tau)$, using $k = -\tau^{-1}\ln\lambda_i$ One important quantity that can be used to describe a binding event is the mean first passage time (MFPT) between two states. The time, τ_{MFPT}, for the system to progress from an arbitrary configuration to a given microstate, say P_i [129], can be evaluated by setting to 0 all rates for transitions leaving P_i, e.g. $T_{ij} = 0, ; \forall j \neq i$. This yields a different matrix, from which the MFPT from any starting state to the absorbing state can be found. When the absorbing state corresponds to the bound configuration, the resulting MFPT can provide the association via

$$k_{on} = (c_{bulk}\ \tau_{MFPT})^{-1} \text{ or} \tag{27}$$

$$k_{off} = \tau_{MFPT}^{-1} \tag{28}$$

where c_{bulk} reflects the ligand concentration for the second-order rate equation. Conversely, a dissociation rate can be computed if the absorbing state corresponds to an unbound configuration, although Eq. 27 reduces to $k_{off} = \tau_{MFPT}^{-1}$ for the first order reaction.

While a single simulation may be limited to tens of microseconds, Markov State Models (MSMs) [130] and related frameworks for dimensionality reduction and deep learning—such as VAMP [131], VAMPnets [132], and TICA [133, 134] have enabled the synthesis of expansive MD simulation data into kinetic models. This strategy effectively couples short, fine-grained MD trajectories to estimate long-timescale kinetics. A conceptually similar approach of spatial discretization is used in multi-scale methods like SEEKR [120] (discussed in Sect. 5.3).

Survival probability When the microstates cannot be reasonably resolved, the survival probability, s(t), provides another quantity to describe the escape of a system from a given state, if it follows a simple exponential decay, e.g. $s(t) = \exp(-kt)$. There are many examples, such as in the gating of ion channels, where a comprehensive definition of microstates as part of an MSM is infeasible. Instead, survival probabilities for canonical states such as open versus closed states can be constructed, for instance, by using single channel currents from a patched ion channel. .

Formally, the survival probability, $s(t)$, reflects the probability that a particle remains in a given state over a given time interval, t. [135]For the simple example of a dissociating complex with a survival probability with a single exponential decay, its integral over $t = 0$ to $t = \infty$ provides an estimate of that decay rate via [135].

$$1/k \equiv \left[\int_0^\infty s(t)dt\right] \tag{29}$$

The survival probability has the advantage of being simple to evaluate for molecular simulations, as it essentially entails evaluating the duration of a complex's association over a large

number of simulation events. In the event that dissociation is well-described as a simple two-state process with mono-exponential decay, Eq. 29 also provides the 'residence time' of the ligand as

$$\tau_{MFPT} = 1/k_{off} \tag{30}$$

This quantity is frequently used as a metric for drug efficacy [136].

Beyond the spherical cow: Anisotropic Reactivity and Dimensionality

Effects of Dimensionality (2D vs. 3D) The dimensionality of the system, such as a 2D plane or a bilayer as shown in (Fig. 2C), versus an open, 3D system, can be a critical factor in shaping the rate or even determining if its value converges. This was elegantly discussed in [27] as a process during which a molecule is adsorbed onto a surface, after which its diffusion within the plane of the surface enhances the rate of sampling the domain. The influence of dimensionality on kinetics demonstrates that for membrane-bound species, 2D diffusion in neuronal membranes increases the re-encounter probability, thereby enhancing association [137] Fig. 2D. with receptors and channels [138]. Such a scenario is reflected in the activity of PI3K, which catalyzes PIP2 to PIP3, the latter of which recruits proteins like AkT to the plasma membrane. The 2D diffusion rate in eukaryotic membranes can be modulated by lipid phase, cholesterol, or obstacles [139]. Relatedly, receptor tethering on the cell surface acts as confinement to enhance diffusion-limited association, like for TNFα ligand with its cognate receptor [140]. Chang and colleagues used BD simulations to investigate the binding of complementary ssDNA strands to ssDNA molecules immobilized on a surface, providing insights into the kinetics of surface-based biosensors [141]. The binding of endogenous, extracellular ligands to GPCRs on the plasma membrane constitutes another paradigm, where a ligand diffuses in 3D to react on a planar membrane that often has a finite-sized reactive site [15] Fig. 2E. Adaptation to BD can be as simple as imposing different boundary conditions (e.g., spherical vs. planar) or using alternative coordinate systems, such as cylindrical coordinates for filaments. However, non-Cartesian coordinate systems are often limited to idealized geometries.

Modern continuum methods and Brownian dynamics simulations with rigid bodies can be used to account for complex protein shapes and electrostatic interactions. For BD, this can be as arbitrary as defining the appropriate geometry. Along these lines, continuum methods commonly use the finite element method for complex protein shapes [142]. These continuum methods define specific reactive patches on a protein or cell surface and can be used to predict diffusion-limited association rates.

4.2.2 Modeling Flexible Solutes

In biologically relevant binding events, such as ligands binding to the deep pockets of a target protein or flexible intrinsically disordered regions (IDRs) binding to their globular protein partners, the conformational flexibility of the binders also plays a key role in modulating the kinetics. Enzymes often undergo conformational changes, some of which are associated with substrate recognition, such as gating, which influence binding kinetics. Meanwhile, IDRs, known for their structural flexibility, are exemplary binders in flexible recognition events. Mechanistically, depending on the nature of these changes, the association can fall in a conformational selection regime, an induced fit regime, or a mixture of these two [19]. Conformational gating or selection, in particular, refers to a mechanism where dynamic regions of a protein transiently expose binding clefts or where ligands adopt compatible conformations, thereby modulating both k_{on} and k_{off} [49] These two models describe different binding mechanisms and manifest differently in the association rate.

While electrostatics, desolvation, and van der Waals interactions play roles similar to those with fixed solutes, flexible molecules introduce the additional complexity of conformational changes, which are vital to the apparent kinetics. In flexible binding events, accurately predicting binding kinetics requires considering the rates of the binding pocket's conformational gating and the intramolecular conformation sampling of the IDRs. For example, in ligand binding to HIV-1 protease [143], the protein samples various conformations where the binding pocket is differentially accessible. This gating phenomenon makes the binding a conformational selection process. In contrast, induced fit has been reported in the binding of p53 to a specific DNA fragment, where DNA binding induces a loop switch in p53 that provides a kinetic advantage over non-specific DNA binding [144]. A mixture of conformational selection and induced fit is often observed in IDR binding. IDRs can retain residual structure (partially folded elements) while remaining highly flexible. Some reports indicate that this residual structure accelerates the coupled binding and folding of IDRs to their targets [145], while others have reported no significant effect [146]. This contrast highlights the case-dependent and complex mechanisms governing the binding of flexible molecules.

BD solutions for kinetics prediction of flexible binding events In biologically relevant binding, atomistic descriptions of the conformational flexibility of real proteins are generally needed. While BD is powerful in simulating the first stage of binding (encounter complex formation), it generally lacks the high resolution needed for the transition to the 'native complex' during the post encounter stage (stage 2 in Fig. 4) where conformational flexibility starts to be important.Generally, binding events involving flexible molecules are determined at the atomistic level from experiment or MD simulations, then used as inputs to BD simulations. Exceptions exist, including early BD studies explicitly considered the flexibility of the active site to achieve more realistic binding rates [147, 148].

Extending the basic NAM framework of BD to account for conformational flexibility to predict kinetics has been done for several model systems. For example, in the BDflex method [149], the authors considered a spherical protein that has a buried pocket. They divided the binding pocket into an exterior and interior space. The ligand's diffusion in the exterior region and its encounter rates at the exterior-interior border were simulated by canonical BD simulations. Meanwhile, in the interior region, the pocket was allowed to switch between a reactive and an unreactive configuration with pre-defined rates. The dividing surface between the exterior and interior region is a radiation boundary that allows BD-sampled flux to proceed into the interior region [149]. This simplified model was capable of capturing the impact of pocket flexibility on predicted association rates.

Prediction of complex ensembles from BD BD and related diffusive dynamics methods can also be used to generate equilibrium conformational ensembles for highly flexible systems in isolation, which can then serve as starting configurations or representative rigid-body states in binding kinetics simulations. This is particularly important for proteins exhibiting conformational gating or intrinsic disorder, where pre-existing structural heterogeneity modulates association pathways and transient encounter complexes. While most applications tend to focus on atomistic-resolution simulations of flexible proteins or IDRs, progress has been made with coarse-grained (CG) models [150]. For example, one study performed extensive coarse-grained Langevin dynamics simulations of IDPs, by introducing only three energy terms (steric repulsive term, attractive hydrophobic term, and electrostatic interactions), the sampled conformational statistics of IDPs agree well with FRET-measured residue-residue distance data [151]. Another study developed an implicit-solvent force field named COFFDROP for Brownian dynamics simulations of proteins and IDRs [152]. This force field derives non-bonded and bonded CG potentials from all-atom MD simulations of all possible two-residue fragments, thus ensuring transferability of the force field. This force field, when coupled with Brownian dynamics, performs well in recapturing the hydrodynamic radii of eight IDRs, including Aβ fragment, ProTα, and α-synuclein, etc. [152]. In a related work, internal coordinates and chained particle positions were propagated using Brownian Dynamics [153] to study the aggregation dynamics of α-synuclein. The α-synuclein protein was represented as a sphere with layered interaction surfaces representing the disordered state versus a spherocylinder with an attractive surface representing the β-sheet state of the protein. A Brownian dynamics model for IDRs based on bead-rod chain dynamics, which includes hydrodynamic interactions and excluded-volume effect, has also been developed [154]. It was used to capture the measured internal dynamics of synthetic $(GS)_n$ IDRs ranging from 11 to 40 residues. All of these approaches can provide important representations of a protein's intrinsically disordered conformational ensemble.

Comparison of kinetics obtained from pure MD approaches While BD is well-suited for the loose assembly of substrate/target complexes, MD-based methods remain indispensable for computing the final assembly of the complex, especially when protein flexibility, solvent organization or ions play critical roles in assembly [27]. As has been discussed in detail by Wang et al [155], physics-based methods include brute-force MD, MD with Markov state modeling, and Biased MD (such as the LiGaMD developed by Yinglong Miao [156]), which usually requiring long simulations [157, 158]. Additionally, unbiased MD simulations performed on the specialized Anton supercomputer, spanning tens to hundreds of microseconds, have been used to identify small-molecule binding pathways for GPCRs [158] and protein kinases [159] without knowledge of the transient encounter complexes. Hence, these simulations provide a continuous description of the binding process, without the requirement of defining a transient encounter interface as is generally needed for BD approaches.

Nevertheless, pure brute-force MD simulations remain challenging for predicting kinetics, as this task requires sampling a sufficient number of binding and unbinding events, which are often rare. Biased sampling strategies are among the most common approaches for estimating the rates of these rare events. Enhanced sampling can be done via biased potentials (umbrella sampling, metadynamics etc.) or weighted ensemble methods [56]. Representative methods include Ligand GaMD (LiGaMD) [156], random acceleration MD [160], metadynamics [161], and WExplore [162].

These methods have been successfully used in computational studies to calculate k_{on} and k_{off} and to reveal detailed binding/unbinding pathways, as summarized in [163].

Newer frameworks dedicated to kinetics prediction are still being developed to cover a broader range of rates. For example, ModBind is a recent MD-based technique that predicts k_{off} values ranging from 10^0 to 10^{-6} s^{-1} [164]. Its strategy involves running simulations at an elevated temperature (e.g. 1000 K) to accelerate unbinding and then reweighting the resulting trajectories to recover the conformational distribution at physiological temperature [164]. As computational resources grow and enhanced sampling techniques mature, predicting kinetics (k_{on} and k_{off}) for ligands binding to medium-sized proteins like trypsin and Src kinase on microsecond timescales using biased sampling is becoming feasible [56], thereby obviating implicit techniques like BD at those scales.

4.3 Transient encounter: BD for Heterogeneous Media

4.3.1 Factors in heterogeneous media

Heterogeneous media often involve mixed solutes and solvent and are characterized by physicochemical properties such as free-volume fraction, charge densities, and enzyme spatial distribution, all of which influence kinetics. Kinetics of binding in heterogeneous media are influenced by several factors. These can include macromolecular crowding, which can both slow diffusion and also trap reactive intermediates to accelerate reactions. Local attractive potentials can further steer substrates, or permit co-localization of enzymes in cellular compartments and LLPS to concentrate reactants and thereby improve reaction efficiency [165] (see also Fig. 7). Together, these factors dictate the local ligand concentration and, consequently, the timescale and amplitude of the signaling response, which can be inferred from Eq. 5. For example, by monitoring the diffusion trajectories of proteins in a cell capable of forming stress granules (SGs), Zhang *et al* [74] showed that under SG formation stimuli, the proteins have a reduced diffusion coefficient (D) compared to normal conditions. Under both conditions, by evaluating the mean-squared displacement versus time (MSD $\propto \Delta t$) relationship, they showed that proteins exhibit a broad range of motion types (e.g., normal vs. confined diffusion), yet the predominant motion type is Brownian [74].

Crowding Protein diffusion in the cytosol often occurs under crowded conditions, which can lead to reduced diffusion coefficients that are often anomalous [166] Macromolecular crowding has a complex effect on kinetics, balancing the hindering effect of increased viscosity with the promoting effect of depletion forces. This macromolecular crowding can profoundly affect kinetics [167]. The effects of crowding in cells are complex, as reviewed by Zhou, Rivas, and Minton [168]: crowding can hinder association by increasing viscosity or promote it by generating depletion forces, in part by modulating the available volume fraction. An example is protein diffusion in the cytosol, which can be depressed and even anomalous due to crowding [166]. Excluded volume effects can also promote association by restricting the configurational entropy to be gained when reactants dissociate [169]. The net impact of crowding on kinetics is a balance between these competing effects [168].

Mixtures also present complex crowding effects depending on the physical attributes of the solutes and their interactions with one another. Crowding impacts not just diffusion but also the conformational landscapes of enzymes and the transition states of a reaction. This can arise from the excluded volume shifting the conformational ensemble toward compact states that may alter binding site accessibility. In other words, excluded volume effects can confine reactants, which can increase the reaction rate, though the crowding can also slow diffusion, which suppresses both k_{on} and k_{off}[168]. For a thorough discussion of simulation with solutes in crowded environments, we refer the readers to Zhou *et al*[168].

Liquid-liquid phase separation The presence of different phases, such as in liquid-liquid phase separation (LLPS) (see Fig. 7), also influences binding kinetics. For instance, reactions can occur in organelles or phase-separated entities like LLPS; eukaryotic cells use organelles like mitochondria and membraneless germline P granules to compartmentalize reactions [170] Fig. 2B. For LLPS, reaction rates are uniquely shaped by the condensate's composition, which can increase local concentrations of favorably partitioned species, exclude competing species, and alter the diffusion behavior of a substrate within the condensate compared to its boundary [171, 172]. Additional considerations are how the physicochemical properties within the condensates themselves can alter catalysis. For example, changes in the solvent viscosity and substrate/active site solvation properties can directly modulate enzyme catalytic rates and specificity [173]. In addition, the condensate environment can influence the nature of the conformational ensembles, which in turn could impact enzyme kinetics, such as that reported for the MORC3 and DDX proteins, where phase separation turns on their enzymatic activities [174, 175].

4.3.2 Modeling reactions in heterogeneous media

Crowded environments Crowded environments motivates the use of effective diffusion coefficients to implicitly represent the influence of crowders on solute diffusion. A simple approximation that can be used to estimate excluded volume effects include the Maxwell-Garnett relationship[176], which modulates the diffusivity as a function of free volume fraction, $\phi \in [0, 1]$ e.g.

$$D_{eff} = D_0 \frac{2\phi}{3 - \phi} \tag{31}$$

for spherical inclusions much greater in size than the solute [176]. In addition, they can be estimated from explicit MD or BD simulations by computation positional or velocity autocorrelation Eq. 11 functions for diffusion crowders [101]

Attempts have been made to use Brownian dynamics simulations to investigate protein-protein association rates within crowded environments, which we depict in Fig. 6D. In one such study, simple hard spheres were used as crowders, and the Brownian motion of two proteins was simulated with pre-calculated diffusion coefficients [177]. This phenomenon poses a challenge for standard BD approaches. The crowding effect was accounted for simply by hard-sphere repulsion among the proteins and crowders. Despite this simplified model, the authors showed that the reduced free volume caused by crowders can enhance association rates by up to two-fold compared to the uncrowded case. Brownian Dynamics can simulate crowding [178] by including explicit crowder molecules as mobile or fixed entities. For systems with fixed crowders, BD can be used to determine an effective diffusion coefficient and calculate corrections for specific interactions. In some cases, the solute's interactions with crowders or the crowders' mobility can give rise to anomalous diffusion [179].

Classical BD assumes an instantaneous decay of the velocity autocorrelation function, which is violated by the memory effects present in anomalous diffusion. This suggests that to accurately model such environments, more general Langevin dynamics frameworks that consider memory effects in the random kernel are appropriate Furthermore, the role of weak, non-specific interactions between solutes and crowders adds another layer of complexity; these interactions generally slow diffusion or lead to off-target binding, though some configurations have been shown to enhance transport. Computationally, a key advancement has been the development of realistic, large-scale simulations of the cytoplasm, such as those by Michael Feig and colleagues, which provide a more accurate emulation of the crowded cellular interior than simpler models using explicit crowder spheres [180]. Note that many IDPs coagulate to form LLPS (such as ABLIM [181]), so here Langevin [182] and BD simulations could be used [183].

Here there is utility in using the concept of 'homogenization'. Homogenization theory provides a procedure to compute effective transport parameters, such as a diffusion coefficient, that is valid for a large problem domain, based on solving a related problem on a smaller, representative domain. [184, 185] This is done by framing the diffusion equation Eq. 12 as a homogenization problem, wherein a related diffusion equation is solved in a representative volume element of the larger domain (Fig. 9B). The solution to that homogenization formulation yields an effective diffusion parameter that captures the influence of crowders on solute diffusion but can be used over the larger domain, without having to explicitly model the crowders. Previous studies have used this approach to model the impact of atomistic-resolution crowders on continuum-scale diffusion coefficients [186, 187], as well as for the diffusion of ATP based on a near-atomistic-resolution representation of the sarcomere [188, 189], which were later shown to agree well with ATP diffusion measurements in skeletal muscle cells [190].

Interestingly, the contributions of crowders can also be reflected implicitly in the PMF itself, which then can be used in tandem with Eq. 19 to predict the association rate. This is helpful, in that the homogenization problem is no longer exclusively reliant on the geometrical boundaries of the crowders, but can incorporate physical factors like long range electrostatics through effective potentials. Further, by changing the form of the Smoluchowski equation (Eq. 19) to resemble a standard diffusion equation, arbitrary mean-field potentials, such as PMFs from atomistic simulations [191], can be reflected in an effective diffusion coefficient [186, 192] appropriate for large-scale simulations.

LLPS modeling BD can be used to simulate co-assembly of solutes into a phase separate from their solvent (Fig. 7). As a recent example, Zhang et al. [193] simulated LLPS dynamics by treating each protein's motion as free Brownian motion within a lattice model. This allows individual proteins to collide and form a condensate or dissociate from it, with probabilities determined by collision and dissociation energy barriers. Both the individual proteins and the resulting condensates are governed by Brownian motion, where the condensate's diffusion coefficient changes with its size. This BD-Lattice model successfully simulated LLPS dynamics, corroborating experimental data.

4.4 Post-Encounter: Hybrid BD/MD Approaches

4.4.1 Modeling the post-encounter event from hybrid BD/MD approaches

While Brownian Dynamics excels at modeling the initial diffusional encounter, simulating the transition to the final bound complex often requires atomistic details best captured by Molecular Dynamics (MD) [194]. Consequently, modern frameworks effectively couple BD with MD-derived information to bridge the gap between the encounter complex and the native bound state [27].

Incorporating MD data for Barriered or Gated Events To resolve binding mechanisms beyond simple diffusion, BD is frequently augmented with atomistic simulations that account for energetic barriers and conformational gating. For equilibrium effects such as desolvation, potentials of mean force (PMF) derived from MD can define the energy landscape governing BD trajectories. Berezhkovskii *et al*[126] utilized this strategy to introduce a model PMF describing the post-encounter event, yielding a correction to k_{on} which could be further refined by incorporating MD-derived position-dependent diffusion coefficients per Hinczewski *et al*[127]. For kinetic effects like conformational gating, BD is often coupled with Markov State Models (MSMs) [143, 195]. If the timescale of the gating motion is slower than that of diffusion, it acts as a kinetic filter, reducing the association rate below the diffusion limit and conferring substrate selectivity based on internal dynamics [196]. Applications of these coupled approaches range from early modulations using simple gating factors [197] to complex systems including the gated binding of drugs to HIV-1 protease [143, 198], the conformation-selective binding of intrinsically disordered calcineurin to calmodulin [53], and the peptide association of BRCA1-BRCT [199].

Directly coupling MD and BD via Milestoning A robust alternative for direct coupling is the use of milestoning theory, as implemented in the SEEKR framework [120, 200, 201]. In this approach, MD provides a high-precision description of binding near the target site (the "post-encounter" region), while BD calculates the diffusional flux to a defined boundary. The macroscopic on-rate is estimated by combining the MD-derived probability that a solute commits to binding (P_{react}) with the BD-derived diffusion rate to the boundary (k_{on}^D):

$$k_{on} = P_{react} \cdot k_{on}^D \tag{32}$$

Their framework was further developed with subsequent code releases [120, 201].

4.5 Multiple Binding Events & Systems: BD treatments

We now discuss the application of BD in the context of multiple, sequential binding events, first within a single complex and then involving multiple.

4.5.1 Cooperativity & Allostery for a single complex

Once a substrate arrives at the transient-encounter hypersurface, its fate is to either diffuse away or to nestle into its final binding mode. It is in this mode that short-range non-bonded interactions between the substrate and target enzyme are optimized, oftentimes through structural rearrangement of the enzyme and the substrate. Much is known about this process and is reviewed extensively in the literature [202, 203]. However, we briefly discuss two phenomena intrinsic to post-encounter binding in the context of the transient encounter: allostery and cooperativity.

Allostery is a common phenomenon where binding at one site influences the affinity for a substrate at another relatively distant site. Cooperativity is closely related, describing changes in affinity for the same substrate, based on preceding binding events, though the sites need not be distant from one another. These concepts are typically approached from an equilibrium perspective, yet an allosteric change can in fact influence binding kinetics. As an example, the members of the G protein-coupled receptor (GPCR) family, the largest group of cell-surface receptors, are inherently allosteric proteins. Agonists bind at an extracellular site to induce heterotrimeric G protein or βarrestin binding on the intracellular side, a distance of tens of Å away. The binding site for the G protein is largely inaccessible until the cognate ligand binds the extracellular side, at which point the G protein may encounter a fully open or gated binding site. Understanding these kinetics of binding, particularly the residence time (Eq. 30), is a key determinant of which downstream signaling pathway is activated [204].

Extending on the earlier example, the pre-assembly of a GPCR with its G protein can affects the affinity for its endogenous ligand [205]. This is an example of 'scaffolding', whereby target proteins are components of a larger macromolecular assembly that tunes substrate affinity. In cardiac myofilaments, for instance, troponin C's calcium binding kinetics are significantly altered when it is assembled into the larger troponin complex, and altered again when assembled with

other components of the thin filament [206]. From an equilibrium perspective, a scaffold might impact affinity by constraining the available conformational states of a protein, disfavoring a low-affinity state, and thereby increasing the relative population of the high-affinity state. From a kinetic perspective, the mechanism is less clear. Ancillary units could alter the energy barrier for binding, perhaps by destabilizing the unbound state, which would increase the rate constant according to Kramers' theory (Eq. 20). Alternatively, if ligand binding is followed by a subsequent binding event elsewhere, the binding of one component could shift the population distribution [207] thereby potentially decreasing the dissociation flux of another component (Eq. 23).

Computational and Analytic Approaches The Hill equation is an analytic approach to characterize cooperative binding phenomena. This equation reads:

$$\theta = \frac{1}{(K_D/L)^n + 1} \tag{33}$$

where θ is the fraction of protein sites that are occupied by ligands, $[L]$ and K_D are ligand concentration and dissociation constants. By fitting the Hill equation to experimentally measured ligand-protein binding curves, the n, which represents positive cooperativity when > 1 and negative cooperativity when < 1, can be obtained.

Another popular analytical model for studying cooperativity and allostery is the Monod–Wyman–Changeux (MWC) model [208]. This model assumes that allosteric proteins are oligomers composed of identical subunits, with each subunit able to pre-exist in at least two conformational states. A small ligand can bind differentially to these pre-existing states. In other words, ligand binding redistributes the equilibrium of the subunit's conformational states, favoring those with higher ligand affinity. A key assumption of the MWC model is that ligand binding to any subunit causes all subunits to adopt the same conformational state distribution. Due to this symmetry constraint, all subunits of the oligomer maintain identical conformational distributions. Consequently, the MWC model is also known as the "concerted model" and has been successfully applied to explain positive cooperative binding, such as oxygen binding to hemoglobin. Recent applications of the MWC model include explaining the positive cooperative binding between allosteric and orthosteric ligands in GPCRs [209].

Besides these analytical models, molecular dynamics (MD) simulation is a powerful tool for revealing allosteric mechanisms in proteins at atomic detail [210]. The advantage of MD for deciphering allostery and cooperative binding is its ability to provide atomistic descriptions of conformational dynamics upon ligand binding, whether to allosteric or orthosteric sites. Furthermore, MD can uncover the communication pathways connecting these multiple sites and identify hidden intermediate states whose stability is sensitive to ligand binding [210]. However, MD generally falls short of sampling sufficient binding and unbinding events to various conformational states. For this reason, while Brownian dynamics (BD) is not traditionally used for insights beyond diffusional encounter, it has immediate potential for predicting binding kinetics to hidden allosteric sites that are unveiled by a bound ligand. Hence, BD could be a cornerstone for estimating the K_{D50} in a Hill equation, or for sites that could contribute to the cooperativity of binding, such as multiple sites for calcium binding in calmodulin.

4.5.2 Signaling cascades and substrate channeling

Cellular responses to stimuli often involve sequential reactions and dynamic enzyme assemblies, which can be modeled computationally. To propagate a signal, cells often respond to extracellular stimuli using a variety of messengers, including ions, ligands, small proteins, or lipids. This can range from an extracellular biomolecule binding a plasma membrane receptor to a response like gene transcription (see Fig. 1, top). This concept underlies the general principle of sequential reactions, where reactant A is converted to B, which is then converted to product C. In some systems, a specific temporal window is employed for negative or positive feedback regulation, or for consensus building among multiple signals [211, 212]. Under certain conditions, this can give rise to oscillatory behaviors [213].

The efficacy of these sequential reactions is shaped by the intrinsic nature of the enzyme, its configuration within macromolecular assemblies (e.g., scaffolds), and its surrounding environment [**Srere1987**]. A key strategy for controlling these pathways is substrate channeling (or shuttling), where coupled reactions occur within the same enzyme or via dynamically assembled enzyme complexes (such as in Fig. 6). Substrate shuttling is a special case of biochemical reactions where a substrate is localized to its active site on a target, and is therefore relevant to both single reactions and multi-reaction systems.

This localization is governed by several physical factors. Electrostatic steering [49, 214] is a well-understood example where an abundance of residues presenting an attractive potential to the substrate helps to guide it to the target; this tends to increase k_{on}. In tandem, steric confinement of a substrate prevents its diffusion away from the binding site, as was elegantly demonstrated

for amino acids and quinones in the PutA peripheral membrane flavoenzyme [215]. While this confinement tends to decrease k_{on}[165], it can improve the efficiency of reactions by decreasing competition with off-target substrates.

In the context of multi-enzyme complexes, the close proximity of enzymes and oftentimes favorable electrostatic interactions with an intermediate prevent significant diffusion of the intermediate away from the reactive centers [216], thereby offering an advantage in accelerating reaction rates [217]. However, the benefit of substrate channeling does not always stem from altering diffusion-limited rates, as catalysis may be reaction-limited [218]. In this case, channeling prevents reactive intermediates from diffusing away to be consumed by other enzymes [219, 220]. This selectivity can be tuned by controlling the distances between reactant sites and the strengths of electrostatic interactions. For instance, enzymes can dynamically assemble to mediate metabolic pathways, such as for glycolysis and oxidative phosphorylation [218], controlling metabolite flux at branch points.

Prime examples of substrate channeling are found throughout metabolism. In the fatty acid synthase (FAS), active substrate channeling occurs between multiple enzymatic domains, enabled by the Acyl Carrier Protein (ACP) domain that covalently binds intermediates and shuttles its cargo [34] (Fig. 2A). Similarly, the mitochondrial electron transport chain is another prime example, where quinone molecules shuttle electrons between large, membrane-embedded complexes [10, 11]. Other examples include DHFR [216] and various glycolysis enzymes [221].

Computational Approaches

Kinetic Monte Carlo and Agent-based models Kinetic Monte Carlo (KMC) is a stochastic simulation method that has been applied to simulate substrate channeling efficiency. For example, this approach was utilized to probe the cascade conversion of glucose to 6-phosphogluconolactone by hexokinase and glucose-6-phosphate dehydrogenase. The study modeled an engineered system where the two enzymes were tethered by a charged linker, which served as a track for substrate transfer [222]. This algorithm relies on evaluating the total escape rate from a given state i as $k_{tot} = \sum_m k_{i,m}$, where $k_{i,m}$ is the transition rate from state i to any accessible state m. The simulation proceeds by drawing a random number $r \in (0, 1]$ to select the next state j', which satisfies the condition:

$$\sum_{m=1}^{j'-1} k_{i,m} < r k_{tot} \leq \sum_{m=1}^{j'} k_{i,m}$$

Following the transition, the system's clock is advanced by a time increment drawn from an exponential distribution with a mean of $1/k_{tot}$ [223]. This procedure iterates to simulate the system's time evolution.

Agent-based models (ABMs), which simulate the behavior of individual molecules (agents) as they react with their environment and with other agents, have also been used to study metabolic channeling in virtual cellular environments [224]. In the work by Klann et al., the conversion of glucose to lactate through four sequential enzymatic reactions was simulated using an ABM [224]. The model considered different spatial arrangements of the four enzymes, either in a linear complex or randomly distributed, and described substrate behavior using predefined effective diffusion constants and reaction rates with individual enzymes. Using this ABM framework, they demonstrated that the final product formation rate is influenced by the enzymes' spatial distribution and that an optimal arrangement exists which maximizes the production rate [224].

PDE-based frameworks The strategies described so far largely apply to particle-centric systems. However, considering ensembles of particles permits the use of continuous representations, namely partial differential equations, to evaluate kinetic parameters we therefore introduce reaction–diffusion equations (Eq. 35), which are continuum, PDE-based representations commonly used to model biomolecular events. A reaction-diffusion system can be used in more elaborate environments that involve complicated geometries or long-range interactions, which may be intractable to solve analytically. For two species, A and $A \cdot B$, that undergo the equilibrium relationship in Eq. 1 we have

$$\frac{\partial A}{\partial t} = D_A \nabla^2 A - k_+[A][B] + k_-[A \cdot B], \tag{34}$$

$$\frac{\partial A \cdot B}{\partial t} = D_{A \cdot B} \nabla^2 [A \cdot B] + k_+[A][B] - k_-[A \cdot B]. \tag{35}$$

As with Eq. 16, the k_{on} of species A binding to its target (B) can be determined by solving for the steady-state of these expressions, then using the steady-state probability to compute the flux along

a reactive boundary via Eq. 19 to yield the association rate. Eq. 34 of course can be extended to incorporate mean-field interactions and in the event that the protein target B and its complex with A are stationary, the diffusion term for $\frac{\partial A \cdot B}{\partial t}$ can be dropped.

Brownian dynamics BD can also be used to study substrate channeling and substrate transfer in parallel with experiment. It can model substrate channeling in dynamic enzyme assemblies by simulating the diffusion of an intermediate product from the active site of one enzyme to the next. This approach allows for the calculation of transfer efficiency as a function of inter-enzyme distance and electrostatic steering. For example, horseradish peroxidase (HRP) and glucose oxidase (GOx) form an enzyme pair where the product of the first (H_2O_2) is the substrate for the second. By immobilizing these enzymes on an artificial scaffold like DNA origami, the impact of inter-enzyme separation on transfer rates can be measured experimentally [225], and these rates can be explicitly modeled by BD [226].

BD simulations have been applied to other substrate channeling phenomena. In the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) system of P. falciparum, for instance, the negatively charged dihydrofolate molecule synthesized at the TS site diffuses to the DHFR site via electrostatic steering. BD simulations revealed the efficiency of this inter-domain substrate transfer and identified the key charged residues that form the electrostatic channel [216], which was followed by a subsequent study for the human isoform by similar methodologies [227]. Similarly, BD has been used to model channeling in the metabolon formed by malate dehydrogenase (MDH) and citrate synthase (CS) in the TCA cycle. These simulations quantified the transfer efficiency of oxaloacetate (OAA) from MDH to CS in the presence of electrostatic channeling effects [221].

4.6 Limitations of BD approaches

Several limitations continue to limit the robust utilization of simulations for binding kinetics. The kinetics of enzymatic processes is a more complex problem than the prediction of binding poses and affinities. Enzymatic reactions that occur in vivo involve multiple substrates, cofactors, and a sequence of elementary steps, making it significantly more difficult to measure and predict overall kinetic fitness [228]. This is compounded by the computational expense of detailed kinetic simulations, especially when solvents are treated explicitly. In addition to the expense, the accuracy can be limited by the quality of the force field parameters used, such as for metals and nonstandard ligands. For these reasons, computational studies are often biased toward protein-small molecule binding because more experimental kinetic data are available for these interactions compared to protein-protein or protein-peptide interactions.

In addition, one of the key limitations of BD is that solvent effects can be difficult to capture, especially close to the solute. Furthermore, a key challenge is the difficulty of creating good implicit solvent models and capturing long spatial and temporal correlations. This could benefit from novel network architectures, such as graph neural networks to learn long-range correlations [229].

5 The Next Frontier

5.1 Introduction

Sections 2- 4 demonstrated how binding kinetics for dilute solutes can be determined using analytic, numerical, and computational methods. However, predictions based on these idealized in vitro conditions are unlikely to translate directly to in vivo contexts, as they neglect the complexity of the cellular environment. Within a living cell, the environment is active and crowded; metabolic fluxes, spatial gradients, and local chemical conditions collectively modulate binding events. Because simulating this complexity is impossible with any single computational method, we now discuss a multi-resolution strategy to approach this frontier. We propose Brownian Dynamics (BD) as a central, unifying interface capable of connecting models across different scales (Fig. 8).

We proceed by outlining several research areas essential for modeling the in vivo cellular environment, beginning with processes involving multiple, sequential binding events (Sect. 5.1.1). In Fig. 8, we illustrate the complex factors, from individual molecular encounters to cellular compartmentalization, that collectively modulate in vivo kinetics. These factors operate over distinct temporal and spatial scales, necessitating a multi-scale computational framework for a complete picture. We emphasize that by integrating multi-event modeling with experimental data, emergent biological insights specific to living systems can be achieved.

The complex binding events in vivo can be categorized into three regimes based on spatial scale: 1) Single binding events: The focus here is on transient encounters, determining how quickly a

binding event occurs and how stable the resulting complex is. At this scale, factors influencing binding rates such as pH, ionic strength, and post-translational modifications, as well as post-encounter effects like conformational flexibility, can be addressed by integrating QM, MD, and BD (Fig. 8). 2) Multiple binding events: For processes like metabolic channeling, BD is especially suitable. Its strength in simulating diffusion-controlled encounters makes it ideal for modeling sequences of binding events where the product of one reaction becomes the substrate for the next. and 3) Cellular scale: At this level, macromolecular crowding and cellular compartmentalization create confined domains. The reduced effective volume, environmental perturbations (e.g., sources and sinks), and surface charges all play critical roles. New simulation methods, such as homogenization techniques and Partial Differential Equation (PDE)-based models, are required to capture these phenomena.

These three scales are integrated in Sect. 5.3 (Fig. 9), where we discuss multiscale modeling as a methodology for connecting biological processes across spatial and temporal scales to capture emergent system behaviors. The section closes with a discussion on leveraging artificial intelligence (AI) techniques to enhance BD-based modeling (Sect. 5.4) and highlights representative applications in areas like drug design (Sect. 5.6).

5.1.1 The challenge of in vivo: cellular environments

Enzyme kinetics is frequently studied in isolated, well-controlled in vitro systems. Similarly, most current applications reflect simplified conditions where targets and substrates exist independent of the cellular environment. In contrast, real-world environments, such as biological mixtures, involve complex media with competing substrates, allosteric modulators, and non-specific interactions. Moreover, it is an important point to recognize that cells operate far from equilibrium, making kinetics of paramount importance. These conditions inevitably constrain proteins within complexes, leading to significant differences in kinetic outcomes. For instance, variations in membrane potential, ionic composition, pH, and macromolecular crowding can all alter the rate of transient encounter complex formation and the composition of the resulting ensemble [230]. Further influencing these factors are covalent, post-translational modifications like oxidation, protonation, and phosphorylation, which can strongly affect the post-encounter state [218]. Accounting for these complex effects in vivo is challenging, thus limiting insights into enzyme function within the cellular environment. Starting with the highly dynamic nature of the cellular environment, it is well established that changes in pH, protein phosphorylation, and intracellular ion concentrations (such as K+) can significantly influence enzyme activity in living cells, yet these factors are rarely incorporated into simulation approaches. We discuss these factors and the role BD could play in accommodating their impacts on enzyme kinetics.

5.1.2 The Need for Bidirectional, Self-Consistent Multiscale Models

A final frontier in addressing these challenges lies in creating novel mathematical and computational frameworks for multiscale simulations. These frameworks must integrate complex considerations, including macromolecular crowding, subcellular compartmentalization, metabolite channeling, and mechanical forces. We argue that the next leap in predictive power requires a paradigm shift away from one-way upscaling towards models built on self-consistency, where information flows bidirectionally between scales Fig. 8. By treating molecular and cellular domains as coupled, we can begin to understand how perturbations propagate and identify the most important emergent modes of the system, such as in metabolic oscillations or G-protein signaling cascades. This approach transforms the challenge into defining important scales to an in vivo system and the robust figures of merit to link and optimize those scales. We propose that BD can play a central role in advancing these efforts (Fig. 9).

5.2 In vivo modeling in stages

5.2.1 Transient encounter in vivo

Cellular changes impacting the transient encounter Brownian Dynamics (BD) simulations are often performed for single-solute systems where factors like ionic strength and pH are held at constant values. However, it is crucial to also explore the cellular context to understand how cellular states (e.g., ATP levels or cell polarization) influence kinetic events in more physiologically-relevant environments. Several physicochemical factors can modulate these kinetic rates. For example, the transient encounter kinetic rate can be affected by macromolecular crowding, which impacts the collision probability between a substrate and its target, particularly when "concentrated" by translocation to cellular compartments or when incorporated in lipid rafts. On a similar note, changes in the local environment, such as the formation of lipid rafts or LLPS regions, can alter binding thermodynamics by localizing substrates and hindering dissociation. Such localization effects can greatly accelerate association kinetics.

Electrostatic interactions and conformational changes influence binding kinetics We see by comparing Eq. 15 to Eq. 18 that electrostatic attraction can accelerate binding kinetics beyond its basal rate, by modifying the reaction PES landscape (Fig. 3). For instance, a pH change that alters a protein's protonation state could increment its net charge, thereby modulating the binding of charged ligands through long-ranged electrostatic interactions. Similarly, covalent post-translational modifications like phosphates could make a protein more negative. Intriguingly, charge changes due to phosphorylation can have opposing effects on the compactness of disordered regions in proteins, depending on the intrinsic charge of the site's neighboring residues [14]. These changes in the conformational ensemble are expected to impact binding site availability and transport toward the bound state. Here, Langevin (flexible) and Brownian (rigid) dynamics are well-suited to treat long-range interactions between a target and its substrate. To connect these molecular-level events to in vivo systems, partial differential equations (PDEs) are helpful tools for modeling environmental changes like pH and surface charge that influence binding kinetics [231]. Given these environment-dependent impacts on conformational ensembles and association kinetics, there is an opportunity to improve drug discovery efforts that have historically focused on optimizing binding affinity and residence time. The existence of multiple protein conformational and catalytic states presents an opportunity to select for more specific functional properties.

Localization and Tethering The study of diffusion-limited reactions in complex biological environments, particularly those involving tethered components, requires a nuanced approach that accounts for physiological accuracy and environmental factors. One example of localization via tethered components includes the diffusion of a disordered region within TnI to bind a cleft unveiled in TnC upon activation with calcium. Typically, studies have modeled tethered components independently of their environment, which would likely yield different behavior in physiologically realistic environments where crowding and electrostatic effects are likely to strongly influence the tethers' properties Fig. 2E). For instance, it is well known that many proteins with intrinsically disordered regions similar to TnI localize to areas like the z-disk in the cardiac sarcomere This localization will, at a minimum, induce complex effects of crowding on the intrinsic association kinetics .

5.2.2 Post-encounter in context of cell state

Just as the cellular environment influences the initial diffusional encounter, it also can profoundly affect the subsequent transition to the final bound state. Cellular signaling pathways can trigger post-translational modifications (PTMs) such as phosphorylation, acetylation, and glycosylation that alter a protein's charge distribution, hydrophobicity, or conformational preferences. These shifts reshape the local energy landscape that is experienced during and after the encounter, modifying the stability of transient intermediates and the height of the transition state that separates them from the final bound state. In addition, the physical local environment like membranes and small-scale geometric constraints in the cell can impact how long the ligand remains near the protein following the initial encounter that associates the molecules. Together these biochemical and structural influences bias the conformational selection process and alter the rate at which the encounter complex can commit to the final bound state and must be taken into account when using BD to simulate the transient encounters available to a diffusing substrate.

5.2.3 Multiple binding events in vivo

A holistic view of cell biology entails considerations of multiple, sequential events that may unfold in parallel. This could be exemplified in the sarcomere by modeling diffusion-influenced binding of Ca2+ to troponin C and its coupling to the kinetics of cross-bridge cycling. This presents two key challenges. The first challenge is to accurately reflect the kinetics of individual 'reactions' within the cellular environment, often while constraining their rates based on established data from in vitro or in vivo experiments, or potentially even other cellular systems [232]. A second challenge is that most studies only consider a small number of reactive centers in explicit detail due to computational or modeling limitations.

From there, the next challenge involves determining free parameters for states of a system that are hidden from direct measurements, such as gating of ion channels that are indirectly determined. Most often, several model topologies can suitably describe the observed data, in turn requiring fitting procedures that evaluate different parameter values and combinations to ensure consistency with experimental data. As a result, these solutions are not expected to be unique and can permit a wide range of values [233] . A variety of optimization techniques have been actively used for this purpose, including genetic algorithms and Bayesian inference. There are also other constraints that may be imposed, such as using the free energy of ATP hydrolysis as a function of pH as a constraint for ATPases [234–236] . Parameter lumping, inspired by Crampin and others, is a related technique whereby fast, equilibrated steps of a kinetic process are merged into macro-states, thus reducing the total number of states in the system and simplifying the

optimization problem.[234, 235] Other constraints may entail ensuring that compartmented or spatially-explicit models yield spatially-averaged values that are temporally consistent with whole-cell measurements [237, 238] To our knowledge, analogous constraints are generally not imposed on the calculation of diffusion-limited reaction rates. Self-consistent models would change this; for instance, macroscale FRET measurements of PLB/SERCA binding could be used to constrain the effective diffusion constant of PLB monomers in a micro-scale BD simulation of their association.

Generally, a compromise must be made between the accuracy of representing proteins (e.g., the detail of the molecular surface or its conformational dynamics) and the number of reactive centers. This trade-off is apparent even when modeling systems comprising only a few reactive centers, such as in the modeling of assemblies like the oxidative phosphorylation complexes like that portrayed in Fig. 2A. In this complex, the kinetics of the respective complexes' dynamics, variations in their relative positions, and the electrodiffusion of the many reactants and products create a combinatorial number of configurations, all of which can influence reaction kinetics and efficiency. Here, BD approaches offer computational savings by modeling individual substrates diffusing between all pertinent reactive centers while assuming that all others are in a non-reactive, steady state. This approach is similar to how continuum reaction-diffusion models handle multiple species, specifically by setting the left-hand side of Eq. 34 to 0. Analogously, many biological processes are often described as a single copy-number event, from which kinetic parameters can be obtained, for instance, by calculating waiting times based on Poisson distributions [239] However, in a biological system, tens of thousands of these events may occur, which motivates using a reduced representation like ordinary differential equations (ODEs) to follow probabilities instead of individual events. This becomes especially important in more complex biological circuits, such as those involving amplification or feedback (Fig. 2D).

It is also important to reflect the contributions of cellular compartments and different phases in contact. Complexes like the electron transport chain in oxidative phosphorylation (Fig. 2A) or chromatin complexes involved in transcription (Fig. 7) involve substrates that partition into different phases. Moreover, substrates involved can shift between these phases, for example, through liquid-liquid phase separation (LLPS) or the aggregation of LIM proteins binding to the sarcomere [181]. In some cases, these phases can be modeled as independent, homogeneous systems, but require careful consideration of interfacial terms like surface tension that will impact solute partitioning. This is related to multi-phase problems where two independent domains are bridged through an interface like a lipid bilayer, for example, through which the transport of material occurs through aquaporins, VDAC, and nuclear pore complexes. The transduction of the binding of an extracellular ligand (GPCR) to promote intracellular G-protein activation is another example of information passed between coupled compartments.

5.3 Multiscale modeling of in vivo systems:

In the preceding section, we highlighted a number of complications that limit the utility of simulations for in vivo systems. Here we highlight the key scales that a typical binding event might entail in vivo, and how linking multiscale models via BD to interpret and predict kinetics could be an attractive solution. At the macroscopic level of cell signaling, continuum models describe species by their average concentrations, either across the whole cell or within specific subcellular regions. At an intermediate scale, coarse-grained models simplify substrate diffusion and binding by representing molecules as single reactive centers. At finer resolutions, the classical physics of molecular dynamics can describe the critical conformational motions of enzymes as they align with substrates. The catalytic conversion itself, however, is a quantum mechanical phenomenon involving the rearrangement of electrons and atomic groups. A single 'in vivo' binding event therefore integrates a vast range of scales, each governed by distinct physical principles.

Computationally, bridging these distinct physical scales is a primary objective. Although robust methods exist for individual scales, developing computationally efficient frameworks to connect them remains a primary research challenge. This process involves defining appropriate fine- and coarse-grained models and establishing procedures to transfer information between them. The reaction–diffusion equation (Eq. 16) provides a mathematical basis for connecting these scales. Here, the goal is to show how the diffusion term ($D\nabla^2 C$) is handled at the continuum level while the PMF term is informed by the particle-based modeling.

We propose BD as a simulation paradigm uniquely well-suited to enable multiscale modeling of molecular systems. Namely, it can serve as a mesoscopic bridge between microscopic, atomistic-resolution systems and macroscopic, continuum-level descriptions. Already, we have discussed how BD is routinely interfaced with molecular dynamics simulations, much in the same way quantum mechanics simulations are hybridized with molecular dynamics (QM/MM) to model enzyme chemistry [240]. At the other end of the spectrum, macroscale cellular processes are commonly described using reaction-diffusion systems, yet the interfacing of BD with continuum models is comparatively less developed. Thus, a key frontier remains in multiscale modeling: the robust linking of particle-based MD and BD simulations with continuum models. Addressing this challenge is necessary for quantitative probes of cellular processes where physically realistic descriptions at

a molecular level are essential. Here, we will illustrate how BD can integrate modeling frameworks that operate at different spatial and temporal scales. The examples highlighted here are successful applications that demonstrate the power of these multiscale approaches and provide a clear path forward for linking with BD.

Exchanging microscopic information with BD

QM:(MM/MD) As a prototypical example of multiscale modeling, the QM/MM approach couples ab initio, quantum chemical models of atomistic electronic structure with classical-particle based molecular dynamics simulations. When applied to substrate/enzyme modeling, it can provide ground state and transition states at stationary points along a reaction pathway and the corresponding zero point energies [240]. We highlight this approach as its key strategy is to identify a boundary acting as an interface between the catalytic site where QM is applied and the classical region across which atom positions and forces are exchanged. (see Fig. 9) Technically, this is done by iteratively solving for an optimal wavefunction in the quantum domain: first, a trial function is computed for a given nuclear coordinate, and then the forces acting on the atoms are used to advance the atomic positions until figures of merit like energies and forces converge. Popular tools that use this method include ONIOM [241] and the generalized hybrid orbital (GHO) method [242]. While useful for predicting reaction pathways and providing estimates of reaction rates [243], QM/MM simulations are generally limited to timescales up to tens of picoseconds, significantly shorter than the millisecond scales required for conformational motions in many enzymes.

BD simulations are generally not directly interfaced with ab initio calculations, though force field parameters and partial charges used for the simulations are commonly obtained from QM calculations, such as RESP [244]. Here, we could leverage the strategy used for polarizable continuum models (PCM) which interface a QM-treated solute with a continuum solvent to provide solvation energies [245]. PCM calculations rely on defining a solute/solvent interface that provides a boundary for continuum solvation modeling based on the electron density predicted from ab initio calculations. Similarly, BD simulations performed in the implicit solvent surrounding a solute could provide substrate/target binding poses as starting points for QM treatments. Specifically, these poses would serve as geometric boundary conditions for QM calculations which in turn generate updated electron densities to reparameterize the electrostatic potential used for the implicit solvent in which BD is conducted. Convergence of the two models could be based on when the BD predicted substrate poses saturate the target surface that is used for the QM based electrostatic potentials.

MD:BD Conventional all-atom molecular dynamics (MD) simulations naturally provide access to much longer timescales relative to QM and QM/MM approaches, with microsecond length simulations now routine . Although MD simulations have been used in tandem with Brownian dynamics (BD) in numerous applications, they have historically been linked in an ad hoc manner, usually by identifying starting conformations with MD with which rigid-body BD simulations are then performed [53] . The recently proposed SEEKR approach offers a more systematic solution that directly couples BD to MD. [201]. Analogous to the QM/MM example, it is based on defining a boundary surface dividing regimes where the two simulation techniques are applied, namely the bound and fully dissociated states. Near the bound configuration, the ligand-enzyme binding is treated using all-atom, explicit solvent MD, and beyond this region BD is used. A key limitation of the SEEKR approach is that the MD/BD boundary is defined a priori, often based on geometric intuition and is usually independent of actual kinetic behavior. However, an adaptive boundary could be defined such that regions where the PMF along the reaction coordinate exhibits significant ruggedness are treated by MD, while the comparatively smooth potential of the outer regions is offloaded to BD simulations. This boundary could be varied iteratively until the k_{on} figure of merit in Eq. 32 reflecting the combined MD/BD contributions achieves convergence betweeen iterations.

CGMD:BD Coarse-grained (CG) particle simulation approaches that use reduced representations of proteins can sample temporal and spatial scales beyond the reach of conventional atomistic simulations. Common strategies include the Martini [246] forcefield for molecular dynamics and COFFDROP [247] for Brownian dynamics, In these models, groups of atoms are represented by single beads and force fields are adjusted to recapture key atomistic-level interactions such as those governing secondary structure. COFFDROP in particular has been used to study molecular association, with applications to IDPs including amyloid β and α synuclein [248]. Most applications of these CG approaches tend to be unidirectional, in so far as they use atomistic resolution data as model inputs to capture coarse-grained properties such as radii of gyration [248] or aggregation [247].

Efforts to backmap or project CG-predicted molecular positions to their atomistic equivalents have been proposed. However, to our knowledge, prominent methods such as the Bayesian inference approach from Liu *et al*[249] have not been applied to simulations of bi-molecular association. In principle, backmapping strategies applied to COFFDROP/BD-based simulations like the IDP aggregation events studied by Ahn *et al*[248] could enable the validation of specific interactions at the atomistic level and be used to refine the CG simulation parameters. Furthermore, partitioning the simulation domain into a region used for atomistic/CG refinement and the other for CG-based Brownian dynamics could be amenable to adaptive boundary approach we described for hybrid MD/BD modeling. Fulfilling this integration would bridge the gap between the efficient sampling of CG dynamics and the structural fidelity required to describe protein protein association. Such a development could be especially useful for proteins with extended, intrinsically disordered regions that are prone to undergoing disordered to ordered transitions during binding [250].

Exchanging macroscopic information with BD

(MD/BD):ODE The examples discussed so far entail mapping between different resolutions of particle based models. However, extending this coupling paradigm to real physiological systems requires interfacing microscopic particle descriptions with macroscopic phenomena described using continuum representations like ordinary differential equations (ODEs). The coupling of molecular resolution models with ODE based systems biology models has gained momentum in recent years, as exemplified by work from Silva *et al* [251]. In that study, the authors bridged simulation paradigms by constructing a potential energy surface (PES) for a potassium channel using Monte Carlo sampling. To connect this thermodynamic landscape to time dependent physiology, they utilized a form of the Smoluchowski equation to compute transition rates along the PES. These rates were subsequently incorporated into a Markov model of channel gating that was then coupled with a ventricular action potential model to link molecular level dynamics to whole cell electrophysiology. More recent applications have similarly combined MD with systems level ODEs. For instance, Teitgen *et al*[252] combined Brownian dynamics and MD to demonstrate how a deoxy-ATP, an ATP analog, impacts myosin/actin association; this was in turn combined with an ODE model to predict impacts on cardiac contractility at cell and organelle levels. Relatedly, our study of ABLIM1 [14] demonstrated how MD and CG simulations of an intrinsically disordered protein can be integrated with an ODE based model of sarcomere contraction. Importantly, these multiscale pipelines replace the reliance on phenomenological fitting common in systems biology with physically derived constants, enabling the quantitative prediction of how specific molecular perturbations, such as mutations or drug binding, propagate to alter organ-level function.

A significant limitation of current ODE-based multiscale approaches is that they typically establish only qualitative relationships between the molecular and ODE scales. For example, a molecular process identified as kinetically favorable may be represented by an arbitrarily increased rate coefficient in an ODE model. A more robust and general strategy would be to replace heuristic parameter estimation with physically derived rate constants. One route toward this goal is to use MD and BD in a complementary fashion. Potential of mean force (PMF) profiles extracted from MD simulations can be used to compute intrinsic rate constants, such as k_{off} and k_{int}, via Kramers' theory. Meanwhile, Brownian dynamics provides a natural means to compute diffusion-limited association rates k_{on}, as implemented in frameworks such as SEEKR. This strategy has been successfully applied in prior work, including studies of adenylyl cyclase where BD simulations were used to calculate the association rates of ATP and G-proteins in crowded environments; the resulting structure-based k_{on} values were fed directly into ODE models to drive simulations of cAMP production [253] . Broad adaptation of integrating BD-based simulations of molecular association with ODE models could offer a substantial quantitative improvement over heuristic rate assignment.

Even rarer, but equally powerful, are top-down constraints, where molecular models are adjusted to satisfy macroscopic dynamics predicted by ODE systems. If, for instance, an ODE model of integrin clustering during cell adhesion [254] predicts a macroscopic rate of change dA/dt required to approach a specific level of tension, that rate of molecular aggregation could be used to constrain BD-based models of their co-assembly, such as from [255]. BD is especially well suited as the intermediate domain for accommodating constraints because it provides a physical context for diffusion, collisions, and reaction probabilities while allowing iterative tuning of microscopic parameters to reproduce a desired macroscopic flux. This tuning generalizes existing practice, where BD/MD encounter criteria, such as steric collision thresholds or reaction probability assignments [111], are adjusted to reproduce experimentally measured rates. By analogy, one could tune the reaction probability or energy thresholds within a BD simulation until the BD-predicted flux matches the macroscopic dA/dt from the ODE.

BD:Continuum Linking particle-based simulations (BD or MD) with continuum representations remains a substantial challenge, though recent applications have made considerable progress. Conventional reaction-diffusion models can accommodate binding or unbinding rates derived from molecular simulations as source–sink terms or mixed boundary conditions, as was recently done by Van Keulen *et al*[253] for a state-based adenyl cyclase model. More systematic bridging approaches using homogenization theory Fig. 9 with molecular-scale structural data have also been used to obtain effective macroscopic parameters like diffusion coefficients [189] or reaction rates [256] suitable for continuum modeling. Strategies such as parameter lumping can consolidate rate equations for processes that equilibrate rapidly relative to the timescale of the continuum system [235], which can help align catalytic mechanisms with atomistic models [236], while reducing problem stiffness and ill-conditioning [257] that can plague deterministic simulations. In parallel, physics-derived interaction potentials representing diverse phenomena including long-range electrostatics or crowding effects can be incorporated directly as mean-field potentials in expressions such as the Smoluchowski equation via the drift term ∇U.

BD can also serve as a dynamic intermediate interface between molecular and continuum models. Ion-channel conduction simulations have provided canonical examples, frequently by representing the channel pore with a PMF inclusive of electrostatic effects, which is used with Brownian dynamics based simulations of ion movement [258, 259]. Various boundary conditions are applied where the pore intersects with a continuum reservoir of ions to ensure ion gradients are maintained, as summarized in [260]. More advanced hybrid BD/continuum schemes [261] support bidirectional exchange by passing particle positions and continuum concentrations through a buffer region, ensuring conservation of mass and momentum. However, many existing MD–BD–continuum couplings utilize static boundaries, which limits one approach to self-consistency across scales. Allowing BD–continuum interfaces to evolve by adjusting boundary placement or permeability to maintain flux continuity would enhance bidirectional information flow and potentially improve computational efficiency.

The Feedback Loop: Closing the Circuit with Differentiable Multiscale Modeling As suggested by these examples, a differentiable multiscale modeling framework centered on the BD interface region could provide a unified strategy for linking particle-based and continuum models. The key advantage lies in establishing a feedback loop that enables information to flow in both directions. This would like rely on backpropagation from macroscopic observables (e.g., tissue-level dynamics) down to fine-grained molecular parameters, then propagating their effects on the microscopic system back to higher-level dynamics. This can be achieved by self-consistently by updating shared properties and dependent variables across scales, though for example, enforcing continuity of chemical potential or conservation of particle flux. Parameters such as the BD–continuum boundary location, energy thresholds, or effective diffusion coefficients could be treated as learnable variables optimized via gradient-based methods to minimize mismatch between predicted and target figures of merit. Such a framework would provide a systematic, data-driven means to refine molecular parameters to align with desired macroscopic behavior, by effectively 'closing the circuit' of multiscale modeling and enabling bidirectional integration between ODE, PDE, BD, and MD representations.

5.4 AI in enzyme kinetics

Multiscale modeling is computationally intensive, a challenge exacerbated by the vast combinatorial degrees of freedom at molecular interfaces. Consequently, scaling such approaches to cell-level representations via brute-force simulation alone is unlikely. Machine Learning (ML) offers a path to scalability by serving as rapid surrogate models that approximate expensive physics-based calculations when sufficient training data are available. In this section, we describe the state of the art in machine learning for kinetics prediction and propose potential directions for coupling ML with Brownian Dynamics (BD) to improve predictions of enzymatic kinetics.

Table 1: ML studies reported on binding kinetics from year 2020 to 2025. All ML models are focused on protein-small molecule dissociation rates predictions.

Model	kinetics	Dataset volume[a]	Complex structure	Descriptors	Performance[b]	Reference
RF	k_{off}	Protein-ligand (501)	X-ray/docking	distance-based protein-ligand atom pairs	$R^2 = 0.60$	2020[262]
RF	k_{off}	Protein-ligand (406)	X-ray/docking	distance based protein-ligand atom pairs	$R^2 = 0.384$	2020 [263]
PLS	k_{off}	p38 kinase (32)	X-ray	protein-ligand interaction energies (EvdW Eeel)	R^2=0.79	2021[264]
RF	k_{off}	PDBbind-koff-2020 (680)	X-ray/docking	distance-based protein-ligand atom pairs	$R^2 = 0.498$	2022 [84]
BNN/RF/SVM, etc	k_{off}	HSP90 set (66)/RIP1 set (35)	X-ray/docking	protein-ligand interaction energies (Evdw Eeel)	R^2= 0.879/0.448[c]	2023[265]
XGBoost/PLS/SVR/KPLS	k_{off}	HSP90 set (132)	X-ray/docking	ligand fingerprint and 3D representations spatially distributed protein-ligand pairs	R^2=0.93	2024 [266]
Mixture-of-Experts based architecture	k_{off}	Protein-ligand (2,773)	n.a.	String inputs (Protein FASTA and ligand fingerprint)	$R^2 = 0.693$	2024 [267]
ProMoBind (pretraining and transfer-learning)	k_{off}	Protein-ligand (684)	n.a.	protein language model (ESM) pretrained ligand 3D representations (Uni-Mol)	$R^2 = 0.522$	2025 [268]

[a]All refer to protein-small molecule interactions
[b]Pearson correlation coefficient (r_p) was converted to R^2
[c]Average performance of eight models on HSP set and RIP1 set, respectively

5.5 While ML models have delivered significant advances in protein structure prediction, their application to enzyme kinetics remains underexplored

Machine learning (ML) has revolutionized the prediction of protein structure, as exemplified by the breakthroughs of RoseTTAFold [97] and AlphaFold [269] in generating accurate models from sequence alone. This success extends to protein design with diffusion-based models like RFdiffusion [270] and to functional interpretation using protein language models such as ESM-2 [271]. These advances provide a powerful foundation, as high-accuracy structural models can directly supply the starting configurations needed for physics-based simulations like BD to predict kinetics. However, the direct application of ML to predict the fundamental k_{on} and k_{off} rate constants of binding remains largely undeveloped. While ML models for predicting biophysical properties like thermostability or binding affinity are well-established [272], their extension to kinetics is rare.

A survey of ML studies on binding kinetics from 2020 to 2025 reveals that only a few tens of studies have been reported. Moreover, these studies focus almost exclusively on predicting the k_{off} rate for protein-inhibitor binding (see Table 1), with no ML models reported for predicting k_{on}. This underdevelopment of kinetic ML models is unsurprising, given the limited volume of experimental kinetic data. Even the reported k_{off} prediction models tend to rely on a few common datasets. These include: 1) The Kinetic Data of Bio-molecular Interaction (*KDBI*) database, which contains experimental kinetic data for protein–protein, protein–ligand, and protein–nucleic acid interactions [273]. Its latest update in 2025 incorporated RNA-protein binding kinetics [274]; 2) The *PDBbind-koff-2020* dataset, containing 680 k_{off} data points for protein–ligand complexes [84]; 3) A dataset of k_{on} and k_{off} values for 104 inhibitors targeting HSP90 [194]. 4) A dataset of k_{on} and k_{off} measurements for 270 inhibitors against 40 kinase targets [89]. 5) The Kinetics for Drug Discovery (*K4DD*) dataset, a resource developed through industry-academia collaboration to advance understanding of drug residence time and its relationship to in vivo efficacy [136].

Beyond limited data volume, the bias toward k_{off} prediction also exists because drug residence time ($1/k_{off}$) is a recognized determinant of in vivo drug efficacy [275], making k_{off}guided optimization pharmacologically meaningful. Furthermore, while ligand-protein bound complex structures provide a good starting point for extracting features to predict k_{off}, predicting k_{on} requires path-dependent features from transient encounter complexes, which are largely elusive.

5.5.1 Opportunities for coupling BD and ML for enzymatic kinetics predictions

The limited volume of experimental kinetic data is a major bottleneck for developing ML models to predict k_{on}. Kinetics prediction is fundamentally more complex than predicting equilibrium structures because a kinetic rate reflects a distinct, path-dependent property rather than a state function, making it a data-intensive problem at the molecular level.

BD can be used to expand the volume of kinetic datasets for ML model training. A key synergy is that molecular simulations have the potential to augment limited experimental datasets. Specifically, BD simulations can be used to generate large-scale kinetic datasets. After calibration against experimental kinetic data, BD can predict association rates for a target receptor, providing the extensive data needed for robust ML model training (Fig. 10). This can be done by first using BD to predict association rates for ligand-target pairs with known experimental values, serving as a calibration stage. Subsequently, BD can be used to predict k_{on} for unseen ligands within the same category, thereby expanding the dataset.

The selection of appropriate features or descriptors is critical for the performance of machine learning models in predicting kinetics. Theoretically, binding kinetics (k_{on} or k_{off}) are determined by both the transition state and the bound state. Therefore, ML features designed to predict these rates should be informed by both states. Most current ML studies on k_{off} use features heavily reliant on bound-state complexes, such as ligand-atom pair distances [84, 262] or interaction energies [265]. This implies that k_{off} values are largely correlated with K_D and that bound-state features are sufficient. While this is supported by one curated database [262], the authors of a kinome-inhibitor kinetics database claim that k_{on} values correlate more strongly with K_D than k_{off} values do [89]. This raises the question of whether bound-state descriptors alone are sufficient. A primary challenge is that current descriptors often under-represent the role of protein dynamics and path-dependence. Although such features can be extracted from MD trajectories sampling binding/unbinding events, this is a computationally expensive strategy unsuitable for high-throughput tasks. BD can alleviate this limitation by efficiently simulating substrate-enzyme encounter complexes. Outputs from BD, such as electrostatic potential maps or encounter complex statistics, could thus serve as valuable input features for ML models (Fig. 10).

Vice versa, ML can enhance BD simulations. ML methods can also be used to directly improve BD estimates or enhance the underlying simulation models. One critical area is the determination of Potentials of Mean Force (PMFs), which are essential inputs for BD. ML methods

are increasingly integrated with PMF determination techniques to improve the accuracy of PMFs derived from MD simulations [276]. An ML model could learn a more accurate PMF or diffusion coefficient, thereby accelerating or improving the accuracy of BD simulations. These refined PMFs can help prioritize sampling in undersampled regions of diffusional space, greatly enhancing BD simulation efficiency (Fig. 10).

5.5.2 Challenges in ML+BD approaches

The development of robust machine learning (ML) models for binding kinetics is significantly hindered by the scarcity of large, high-quality, and standardized experimental datasets for kinetic rate constants (k_{on} and k_{off}). In contrast to binding affinity (K_D, K_i), where public databases are relatively extensive, kinetic data is sparse. This paucity presents challenges regarding the management of incomplete datasets and limits the statistical power of models. Moreover, existing kinetic data are often heterogeneous, having been measured under varying experimental conditions, which complicates the curation of consistent training sets.

This scarcity of high-quality data exacerbates issues regarding model validation, particularly information leakage. A common pitfall in biological ML arises when models are trained and tested on non-independent datasets. Without rigorous splitting, models may simply memorize sequence patterns absent in unseen targets rather than learning generalizable principles [277]. Consequently, high-quality datasets are essential for implementing homology-aware splitting, where training and testing sets are divided to ensure no close evolutionary relationship exists between them. This ensures the model ascertains transferable physicochemical rules rather than dataset-specific biases.

Beyond data availability, predicting k_{on} is intrinsically difficult due to the complexity of the underlying physics. These difficulties include modeling implicit solvent effects and capturing long-range spatial and temporal correlations. Addressing these physical complexities often requires advanced network architectures, such as 3D Convolutional Neural Networks (CNNs), capable of identifying patterns in complex spatial data. Such approaches typically partition the protein interaction space into voxels containing physicochemical properties like electrostatic potentials and hydrophobic character; this discretization allows the network to identify long-range correlations and geometric patterns .

Finally, these challenges are magnified when moving from binding to more complex biological processes. Catalysis, for instance, presents a more substantial challenge than simple binding because enzymatic reactions involve intricate fitness landscapes, multiple substrates, cofactors, and sequences of elementary steps. This complexity makes predicting the overall kinetic fitness for catalysis significantly harder than for simple binding events [228].

5.6 Applications

We next outline potentially applications of multiscale modeling using BD as an interface. Ideally, ML could be used in context with the multiscale modeling, to reduce computational expense of redundant calculations or for the identification of pertinent degrees of freedom for integrating interfaced models.

5.6.1 Drug Design and Systems Pharmacology

In drug discovery, binding kinetics are critical determinants of a drug's potency and duration of action, with a faster k_{on} and slower k_{off}(i.e., a longer residence time) generally being advantageous. For instance, it is increasingly recognized that the residence time of the drug (see Eq. 30) on its target is a critical determinant of its pharmacological effect in a dynamic, open physiological system [56]. Sustained target inhibition is believed to provide a more durable therapeutic, making kinetics a key consideration in modern drug development [278]. For example, a long residence time (slow k_{off}rates) can be more important for efficacy than binding affinity in many kinase inhibitors, such as those targeting the PI3K pathway [279].

Once the cellular state is well-characterized, multiscale modeling could be applied to optimize the design and delivery of therapeutics. An in vivo model could define these critical temporal windows, thereby placing explicit constraints on the necessary k_{on} and k_{off} for a successful therapeutic. Since many drug-target association events are reaction-limited (not diffusion-limited), the binding process could be governed by conformational selection or induced-fit mechanisms, or potentially even rates of rebinding a drug target , making these phenomena prime targets for computational design. Computational models in this context provide a framework for designing drugs that target specific functional states, such as intermediate states of ion channels, or by promoting allosteric trapping of desired conformations. BD is particularly well-suited to resolve the kinetics of binding to such cryptic or transient sites, thereby enabling the design of pathway-specific drugs that tune residence times and, ultimately, therapeutic efficacy.

A differentiable, multiscale approach elevates this paradigm beyond optimizing a single parameter like affinity by allowing for the co-optimization of multiple, interconnected properties under

constraints. For instance, one could design a drug that not only has a long residence time but also exhibits favorable transport properties in a crowded milieu and avoids off-target interactions. Because biological fitness landscapes are often rugged, methods like genetic algorithms could be employed within this framework to explore alternative mechanistic solutions for achieving a therapeutic effect, moving beyond the single, obvious target and offering a powerful tool for holistic drug and delivery system design.

5.6.2 Peptide design for dynamic processes

A BD-centric framework for integrating molecular-level alterations with cellular-level outcomes could be invaluable for probing and designing signaling pathways. As an example, there are cell lines transiently expressing RyR and SERCA that induce spontaneous calcium oscillations [280, 281]. Within this system, experimental techniques, like FRET, can be used to assess the binding kinetics of regulatory proteins (e.g., PLB to SERCA), and these kinetics could then be used to define an ODE-based model of cellular transients. Brownian Dynamics here offers a powerful extension by predicting these binding kinetics, for instance, by representing the PLB-SERCA interaction as a two-dimensional diffusion process within the membrane. In principle, BD simulations could capture both the diffusion and the diffusion-influenced association of membrane-bound targets.

This setup provides an ideal scenario for multiscale approaches to bridge micro and macro scales. The connection shows how understanding conformational kinetics could guide the rational design of therapeutic peptides that restore calcium homeostasis. The concept of targeting protein conformations can be generalized by designing drugs to 'trap' specific protein conformations, for example, by modulating RyR ion channel gating kinetics [282]. Conversely, a protein's gating rates can be differentially affected by various ligands, leading to an equilibrium shift in binding site conformations that significantly alters the ligand unbinding kinetics (k_{off}), as seen in the FabI enzyme of bacterial fatty acid synthase (FAS) [283] and the HSP90 protein [266]. Given that BD's core strength is its ability to bridge atomistic conformational changes to mesoscale concentration fields by resolving substrate and target diffusion in crowded cellular environments, BD not only complements continuum models of diffusion but also enables mechanistic predictions of how local molecular interactions scale up to cellular signaling outcomes.

5.6.3 Extension to Other Biological Systems

In biological systems, reaction–diffusion processes at the molecular scale often have direct analogues at longer time and larger spatial scales involving higher-order entities like macromolecular complexes or even cells. For example, isolated cells exhibit Brownian-like motion and directed migration along chemokine gradients, a process that parallels how molecular species are modeled moving within a mean-field potential. Over the years, it has also been used to study viral capsid [284] and actin filament assembly [285]. Likewise, at the population level, collective cell migration during processes like wound healing can be described by Keller–Segel equations, which closely resemble the Smoluchowski formalism for interacting Brownian particles [286]. This apparent scale invariance suggests that the fundamental concepts of Brownian Dynamics are transferable from molecules to larger assemblies. This correspondence opens opportunities to apply diffusion-based modeling across multiple scales of biological organization, translating insights from molecular signaling to tissue-level dynamics.

5.6.4 Applications: Model validation and experimental alignment

Lastly, the predictive power of computational models is realized when rigorously validated and constrained by experimental data. Leveraging detailed experimental techniques can significantly inform the timescales of key biomolecular events modeled via Brownian dynamics. In this regard, there is a critical need for aligning experimental and computational datasets. Such alignment priorities were raised in a review of aligning molecular crowding experiments with biomolecular simulations [287], but only grow in complexity when considering molecular association events. Here, Bayesian inference has served a prominent role in integrating diverse data sets, from time-resolved X-ray crystallography, NMR, FRET, to stopped-flow kinetics, to refine simulation parameters to align with experimental observations. A recent study demonstrated how Bayes inference could be used to parameterize ODE-based systems biology models from live cell fluorescence imaging data[233]. This suggests the potential feasibility of such methods for parameterizing more detailed simulations, such as BD, although the intense computational demands of Bayesian methods will limit brute force implmentations.

6 Conclusion

In this review, we have discussed fundamental complexities of biomolecular binding kinetics, from the classical principles of mass action to the stochastic intricacies of the living cell. We have emphasized that the kinetic rate constants (k_{on} and k_{off}) are not merely fixed parameters, but dynamic determinants of cellular function, pharmacological behavior, and therapeutic efficacy. Central to this discussion is Brownian Dynamics (BD), which we identify not simply as a simulation tool, but as an indispensable mesoscopic bridge linking atomistic molecular dynamics with macroscopic continuum models.

We have further examined how the idealized two-stage binding process comprising the diffusional encounter followed by molecular recognition is profoundly modulated by the cellular milieu. Factors such as macromolecular crowding, phase separation, and electrostatic steering do not merely perturb binding events, but reshape the underlying energy landscapes that govern them. Although hybrid BD–MD approaches have emerged as powerful strategies for capturing these multiscale pathways, forward simulation alone is unlikely to be sufficient to address the full complexity of biological environments.

The next frontier in kinetic modeling lies in moving beyond simulation toward inverse design. Because the in vivo environment encompasses an overwhelming number of interacting parameters, the field would benefit from a shift toward self-consistent, bidirectional, multiscale models. We propose the development of differentiable frameworks with feedback loops in which macroscopic continuum fluxes constrain microscopic BD transition probabilities. Such an approach would allow biological systems to be simulated as coupled, optimizable entities across scales, opening new avenues for both understanding and engineering complex biological processes in vivo.

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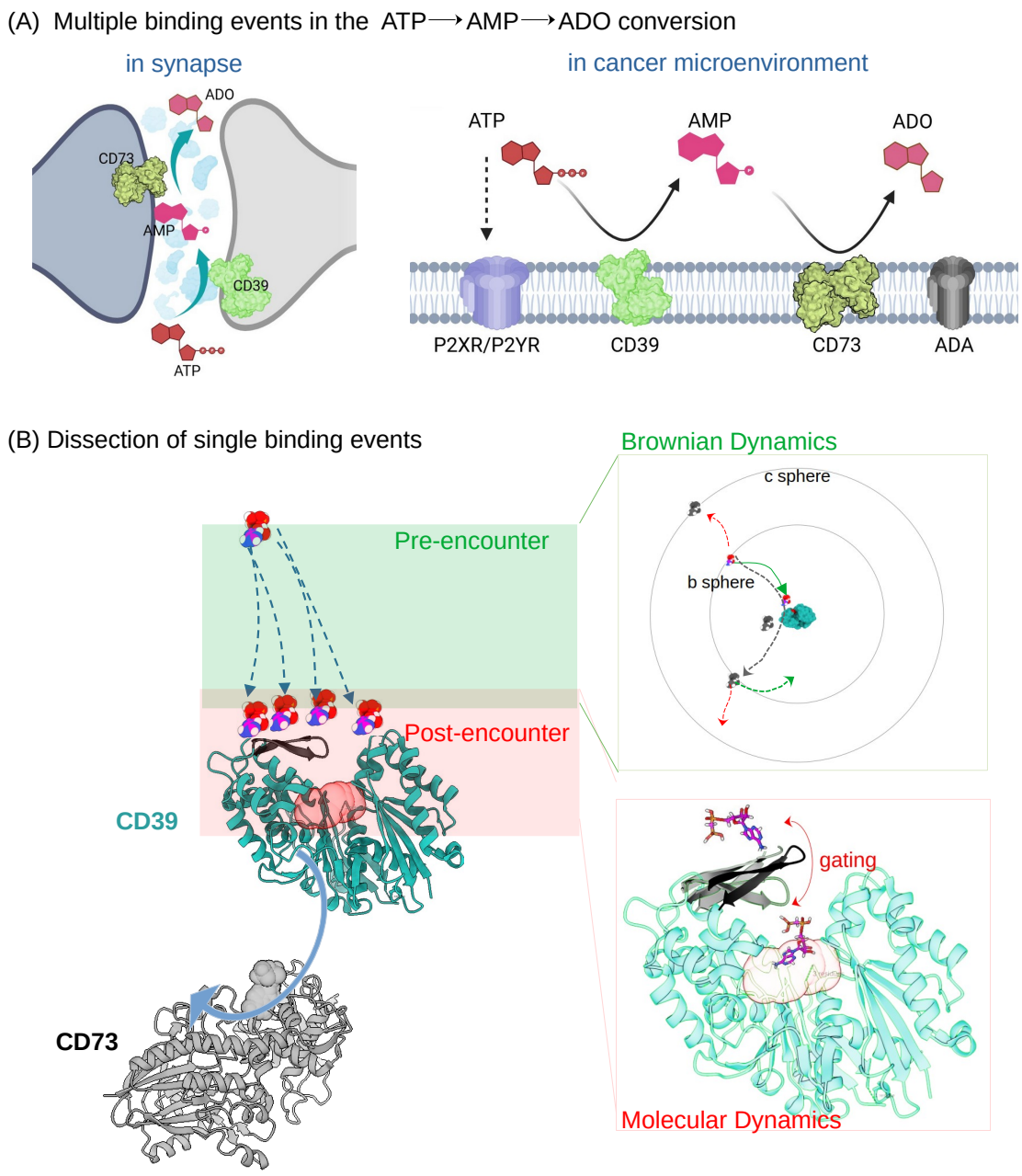

Figure 1: (A) Sequential reactions or multiple binding events are of great biological relevance. Such as the ATP(ADP) to AMP to ATP conversion that are sequentially catalyzed by membrane-embedded CD39 and CD73 enzymes, plays an important role in synapse signaling[165] as well as in cancer immunosuppression [36]. (B) Dissection of the basic single binding events into to the pre-encounter stage and the post-encounter stage. In this review, we describe how Brownian dynamics simulations are well-suited for modeling the diffusive encounter rates at the transient-encounter stage. It does so by incorporating long-range electrostatic interactions, solvation effects, hydrodynamic effects, and the diffusion behavior of rigid-body biomolecules In the post-encounter where conformation flexibility, intra-molecular dynamics (such as binding site gating) and solvation changes serve important roles in molecular recognition, molecular dynamics simulation techniques are generally used to capture these subtle behaviors. .

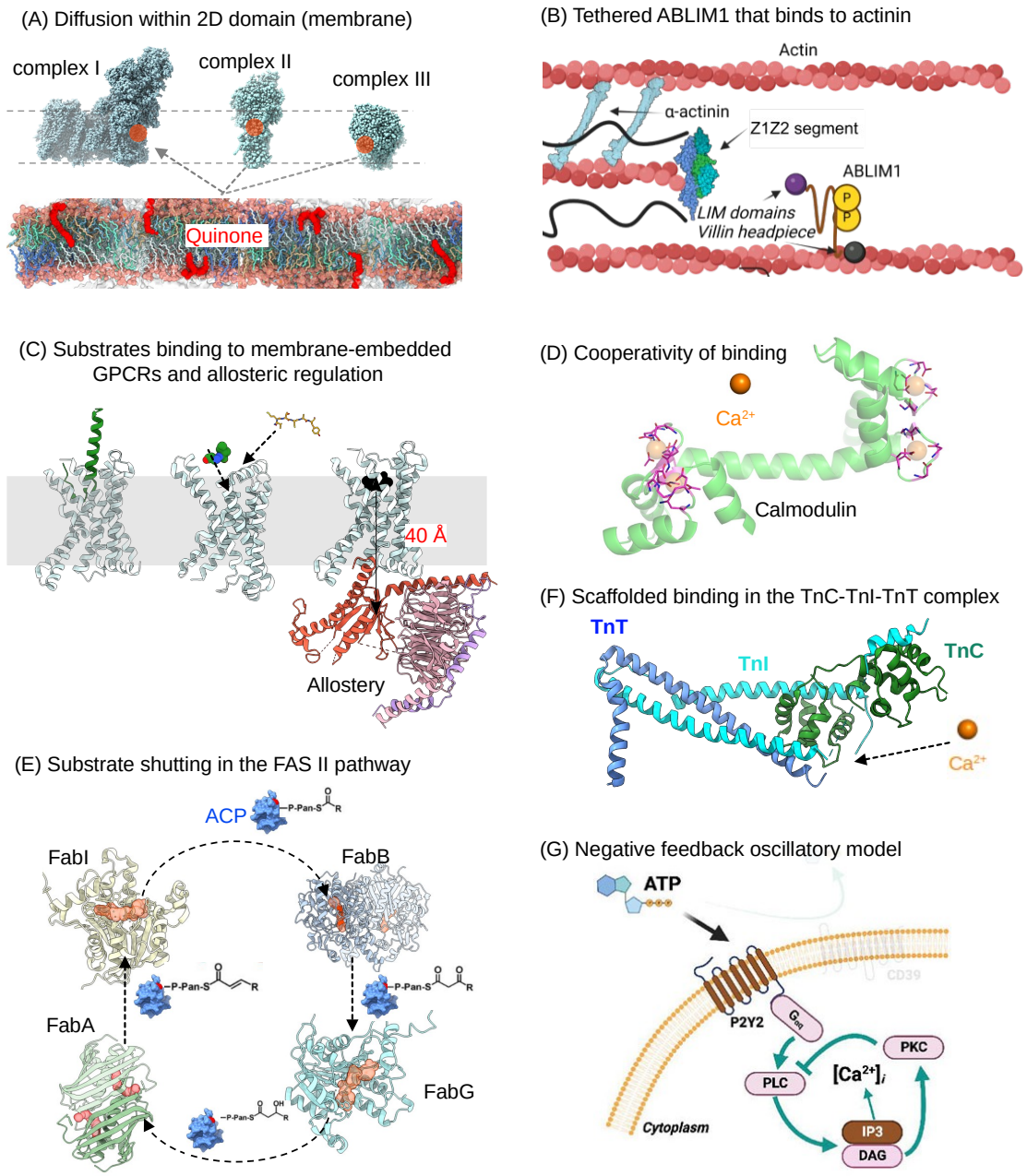

Figure 2: **Complex environments of binding events, scales, and feedback.** Although conceptually the binding events only involve two binding partners, in biological systems, binding events are much more complex as they occur in special environments. (A) Substrate diffusion in a special medium such as membrane, shown is the quinone molecules that diffuse within mitochondria inner membrane and serve as charge carriers to transfer electrons among respiratory complexes[10, 11]. (B) 'Tethered' actin-binding LIM domain protein (ABLIM) (@2025 Sun et al. originally published in [14]), (C) Agonists from the extracellular environment bind to the cell-membrane embedded GPCR receptors, and ultimately promote the binding of intracellular proteins via an allosteric response. (D) Cooperative binding between Ca^{2+} ions and calmodulin, a protein that has four Ca^{2+} binding sites called the EF-hands [31]. (E) Substrate shuttling between enzymes, as is typically seen in the FAS II pathway where ACP protein carries intermediate molecules shuttling between enzymes via diffusion [34]. (F) Scaffolded binding of TnI-TnC-TnI that is regulated by Ca^{2+} ions [35]. (G) Oscillatory, negative feedback shown is phospholipase C (PLC), protein kinase C (PKC), and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) cycle adapted from [289]. .

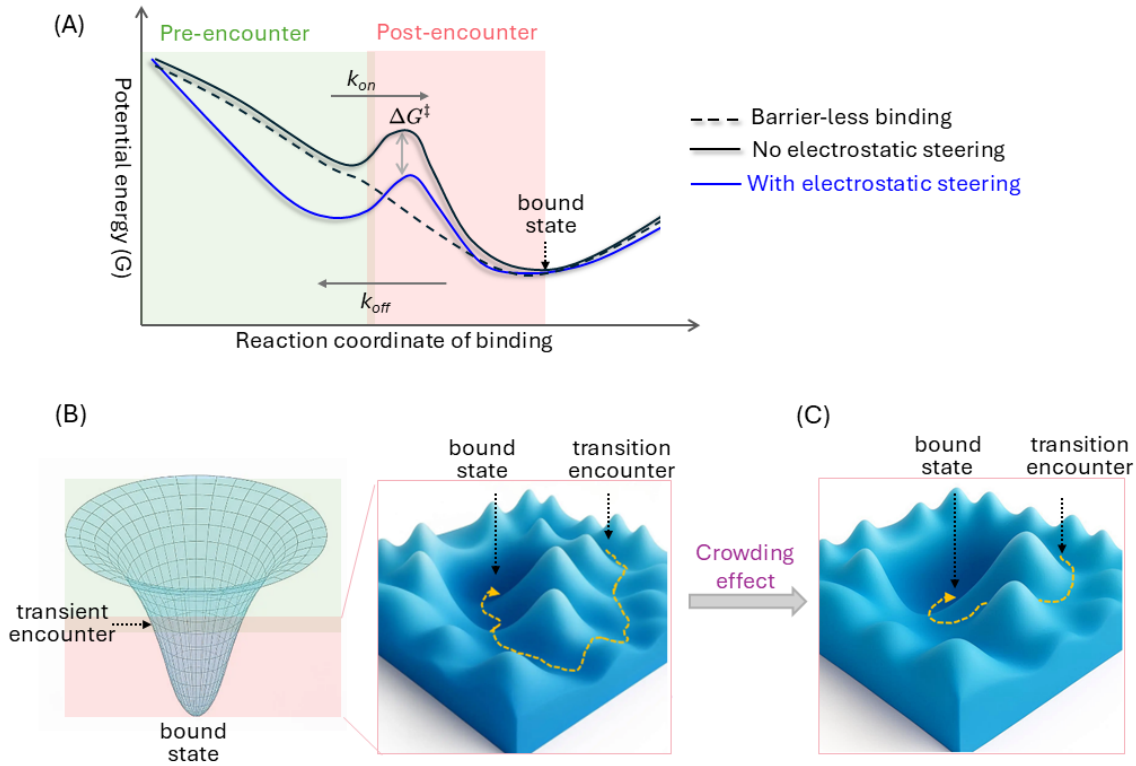

Figure 3: **Energy landscape view of biomolecular binding events.** (A) The overall potential energy of the system decreases throughout the binding process, from the pre- to post-encounter stages, until the bound state is reached. The thermodynamic favorability of the bound state makes binding a spontaneous process. However, the binding rates (k_{on} and k_{off}) are determined by the heights of the energy barriers, not merely by the stability of the final complex. During the pre-encounter stage, biomolecules are often guided by long-range electrostatic interactions that accelerate the formation of encounter complexes. (B) Binding follows a funneled energy landscape, with the native bound state at the global minimum. The intrinsic flexibility of biomolecules creates a rough binding funnel, featuring numerous metastable intermediate states between the initial encounter and the final bound state. In a realistic cellular environment, macromolecular crowding can be conceptualized as altering the distribution and stability of these intermediate states, thereby modulating the overall binding kinetics. .

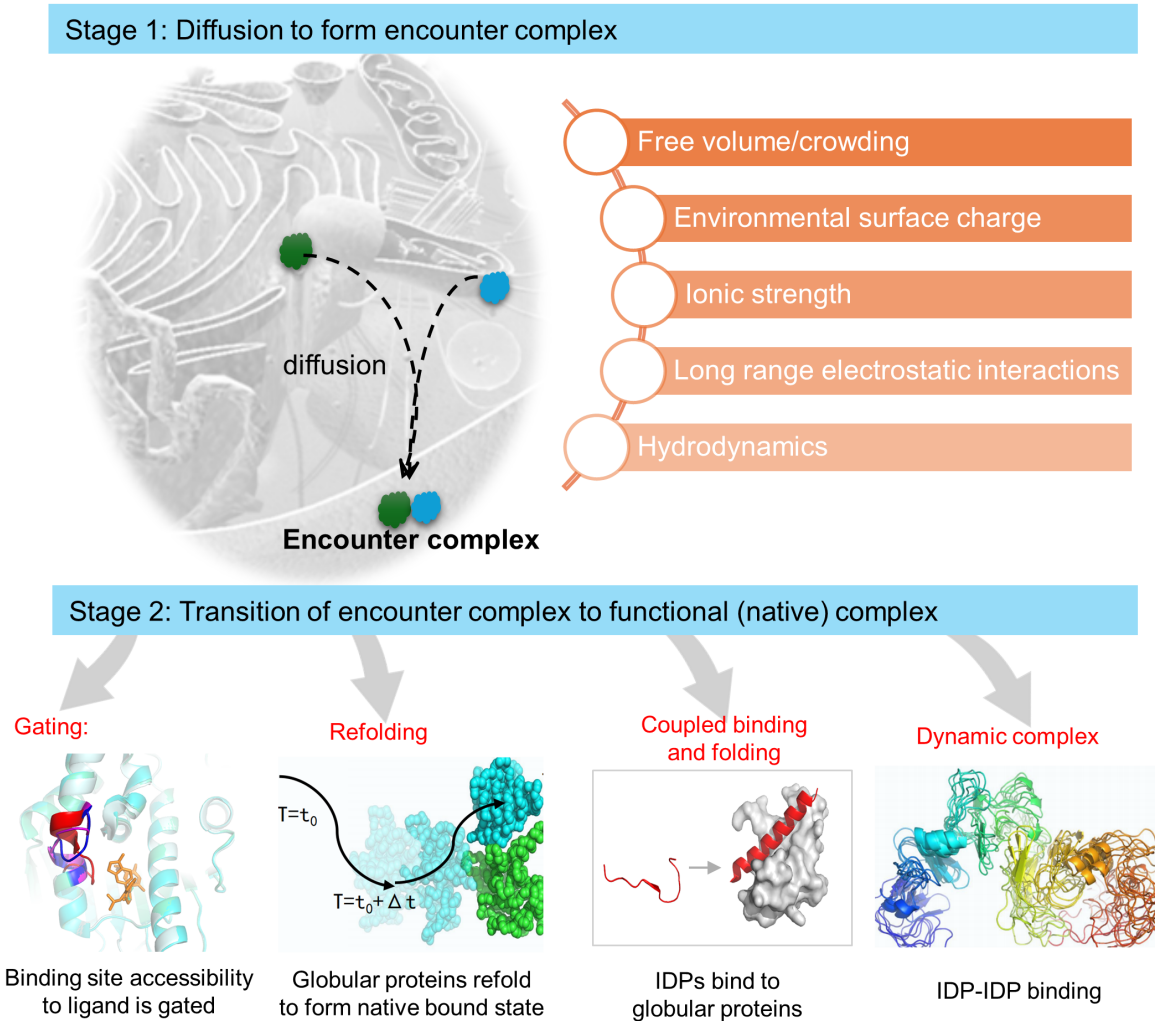

Figure 4: Biomolecule binding is frequently described as a two-stage process (Fig. 3C). The first stage involves the formation of an encounter complex through diffusion, often guided by long-range electrostatic interactions, with typical rates of 10^5 to 10^6 $M^{-1}s^{-1}$. The second stage is the transition from this encounter complex to the final, functional complex, a process whose rate is determined by molecule-specific factors. The gating example is seen in the inhibitors-HSP90 binding [266] where the binding site's accessibility to ligands is controlled by the conformational states of a lid that can transition between loops and α-helix [266]. The refolding of transient complex to the final bound state can be seen in the p53-ASSP protein-protein binding where simulation-captured encounter complexes exhibited structural fluctuation relative to the initial contact pose, evidencing the occurrence of refolding [290]. The coupled binding and folding is a paradigm of recognition mechanism between intrinsically disordered protein (IDP) and its globular target, where the IDP gains secondary structure upon forming the final bound state[157]. Binding can also be in the form of fuzzy complexes, typically seen in IDP-IDP binding, such as the H1–ProTα complex[291], where the conformational entropy plays a key role in determining the binding free energy. Modern structural and AI offer compelling possibilities for probing transient states. For instance, preliminary concepts for encounter geometries can be generated from programs like AlphaFold-Multimer [96], then refined with BD simulations. In parallel, generative AI models could be trained with emergent data from time-resolved x-ray or NMR to capture transient structural data. .

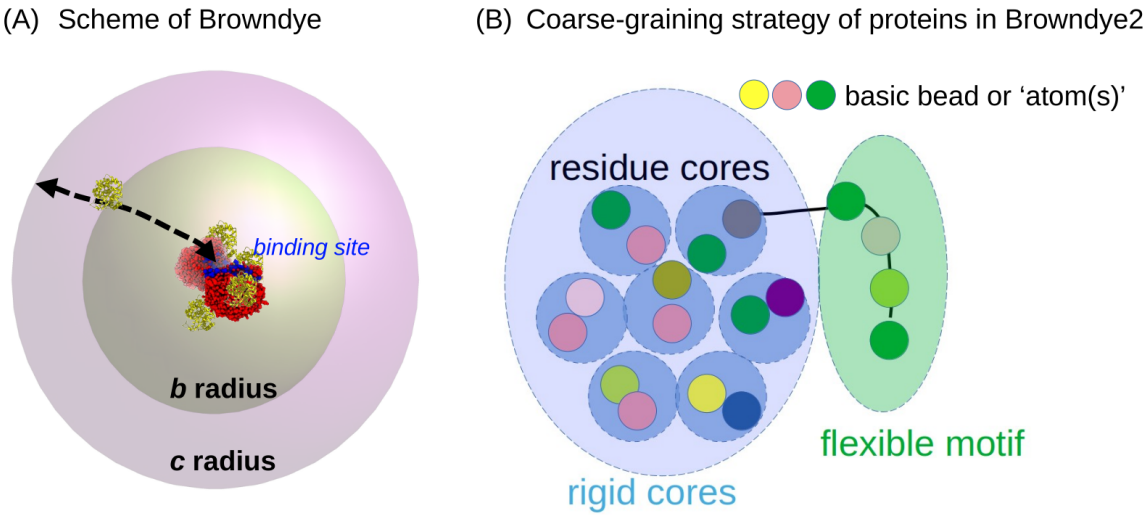

Figure 5: The NAM framework [4] that utilizes BD to calculate the k_{on}, and this framework has been implemented in the software Browndye and Browndye2 [111]. (A) The b sphere and c spheres that mark the starting position of the binders and the boundary where the binder reaches "escape" boundary. By setting the reaction criteria that constrains the ligand to bind to a pre-defined binding site, the effects of conformation selection can be accounted in the calculated k_{on} via Browndye. (B) The Browndye2 utilized a special coarse-graining strategy to achieve efficient simulation of diffusional encounter statistics as well as to account for conformational flexibility during binding by treating parts of the binders as flexible chains/motifs. .

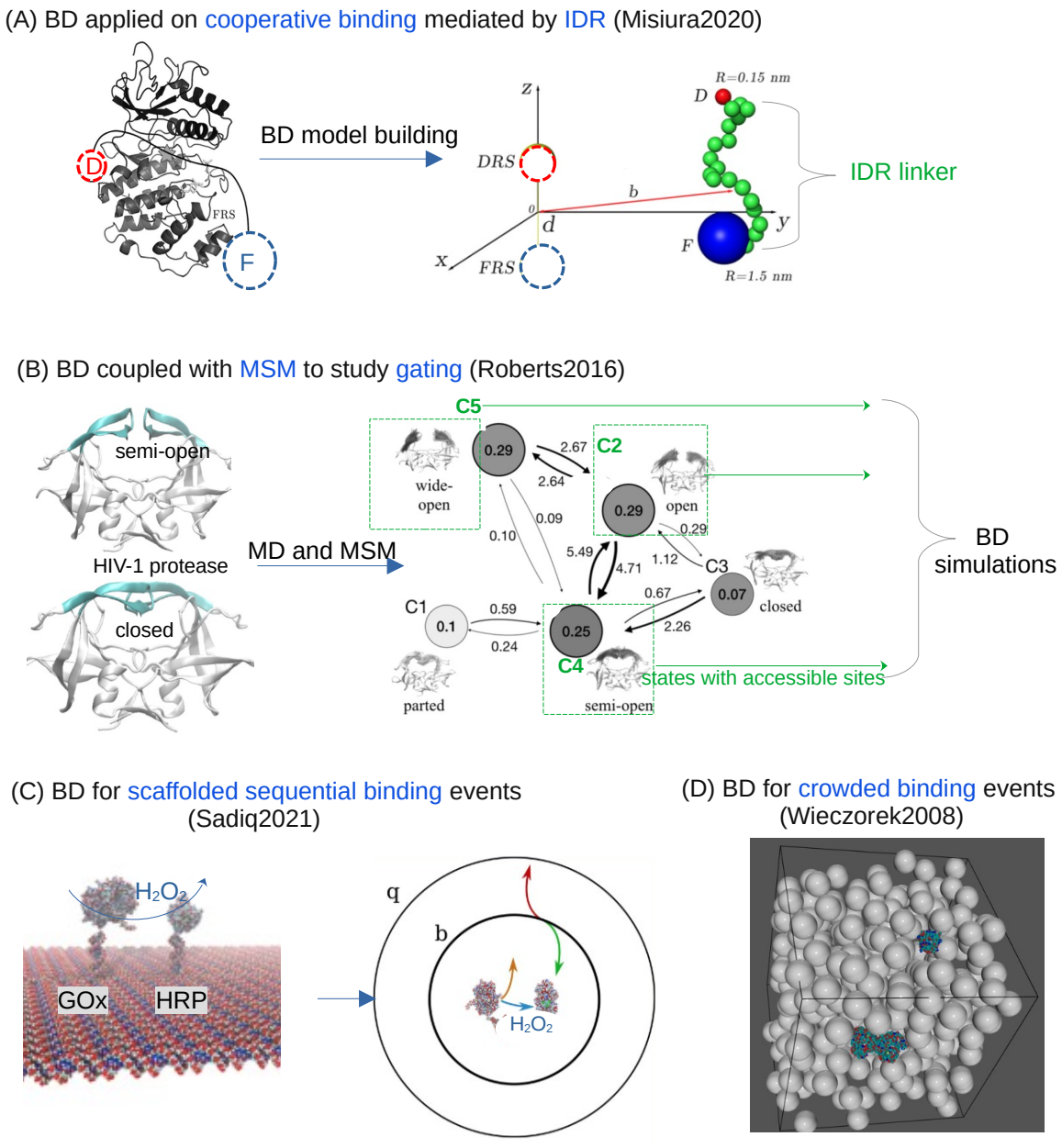

Figure 6: **Exemplary BD applications for calculating binding rates under biologically complex conditions.** (A) Multi-site binding involving the ERK2 enzyme. ERK2 possesses two binding sites for the two domains of the EtsΔ138 protein, which are connected by an IDR linker. BD simulations by Misiura *et al* demonstrated that this IDR tethering accelerates association rates by 3- to 4-fold [292]. Adapted with permission from [292]. (B) Hybrid BD/MD/MSM approach for ligand binding to HIV-1 protease. Sadiq *et al* coupled BD with MD and MSM to accurately calculate binding rates [143]. MD and MSM first identified the binding-site-accessible microstates of the ligand, which were then used as starting points for rigid-body BD simulations. The final binding rate incorporates both the BD-simulated diffusion rate and the conversion rates between binding-site-accessible and inaccessible microstates. Adapted with permission from [143]. (C) BD simulation of scaffolded sequential binding. BD has been applied to study sequential reactions, such as the transfer of H_2O_2 between the GOx and HRP enzyme pair, where H_2O_2 is a product of GOx and a substrate for HRP. Simulations elucidated the impact of enzyme separation distance on the H_2O_2 transfer rate [226]. Adapted with permission from [226]. (D) BD for protein association in crowded environments. BD simulations have been used to investigate how macromolecular crowding affects protein-protein association rates. For example, Wieczorek *et al* modeled crowders as hard spheres and simulated the diffusion of two proteins using pre-calculated diffusion coefficients to study this effect [177]. Adapted with permission from [177]. .

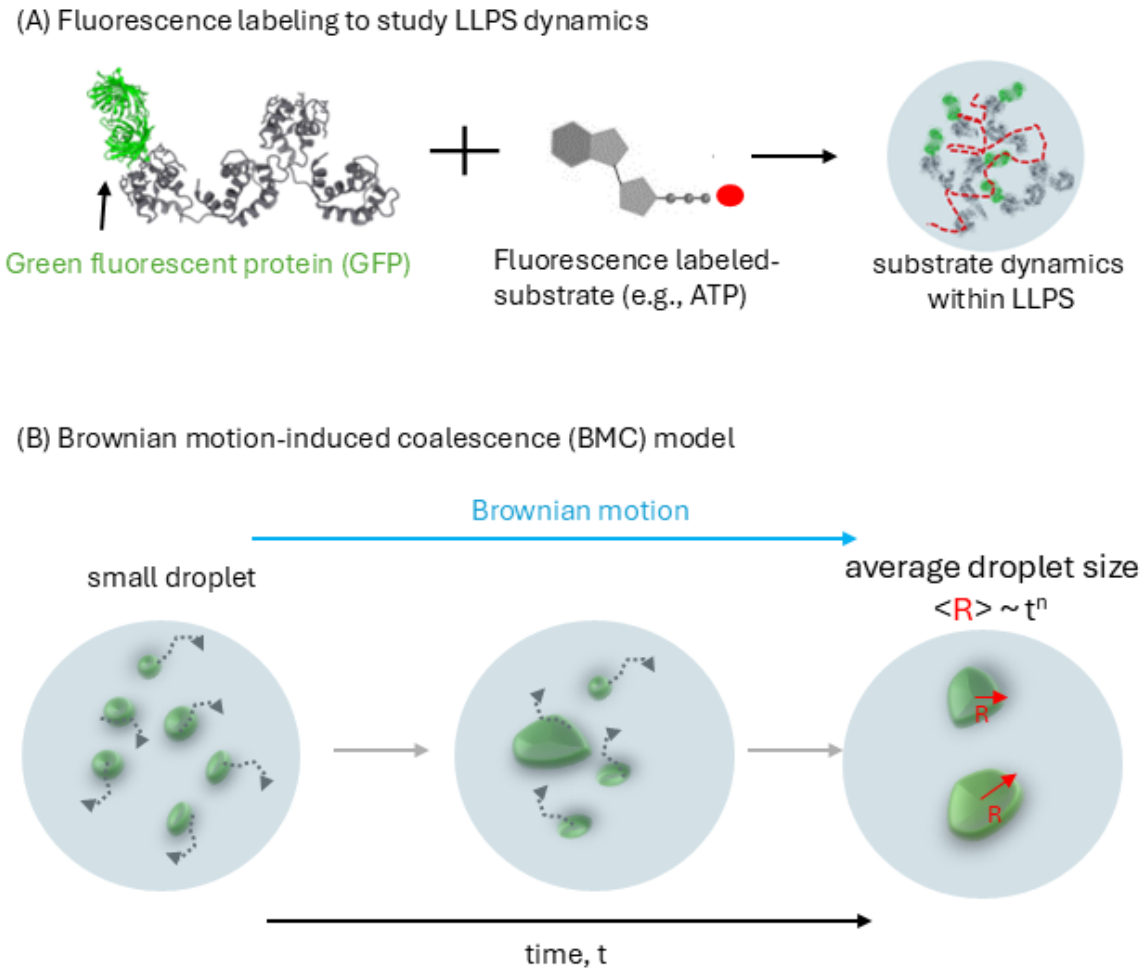

Figure 7: **Brownian-type motions dominate LLPS dynamics.** (A) Experimental observation via imaging. Imaging techniques are used to capture experimental data on LLPS dynamics, such as the diffusion trajectories of individual molecules [74] or the time-dependent change in droplet volume [293]. (B) Modeling with Brownian motion-induced coalescence (BMC). The BMC model [294] serves as a fundamental framework for explaining LLPS dynamics in living cells, including the formation of nuclear bodies [293, 295] and transcription factor condensates [76]. Brownian motion provides a foundational model upon which additional potentials (e.g., for collision or dissociation) and modeling techniques (such as lattice models) can be integrated to explain case-specific LLPS data [193].

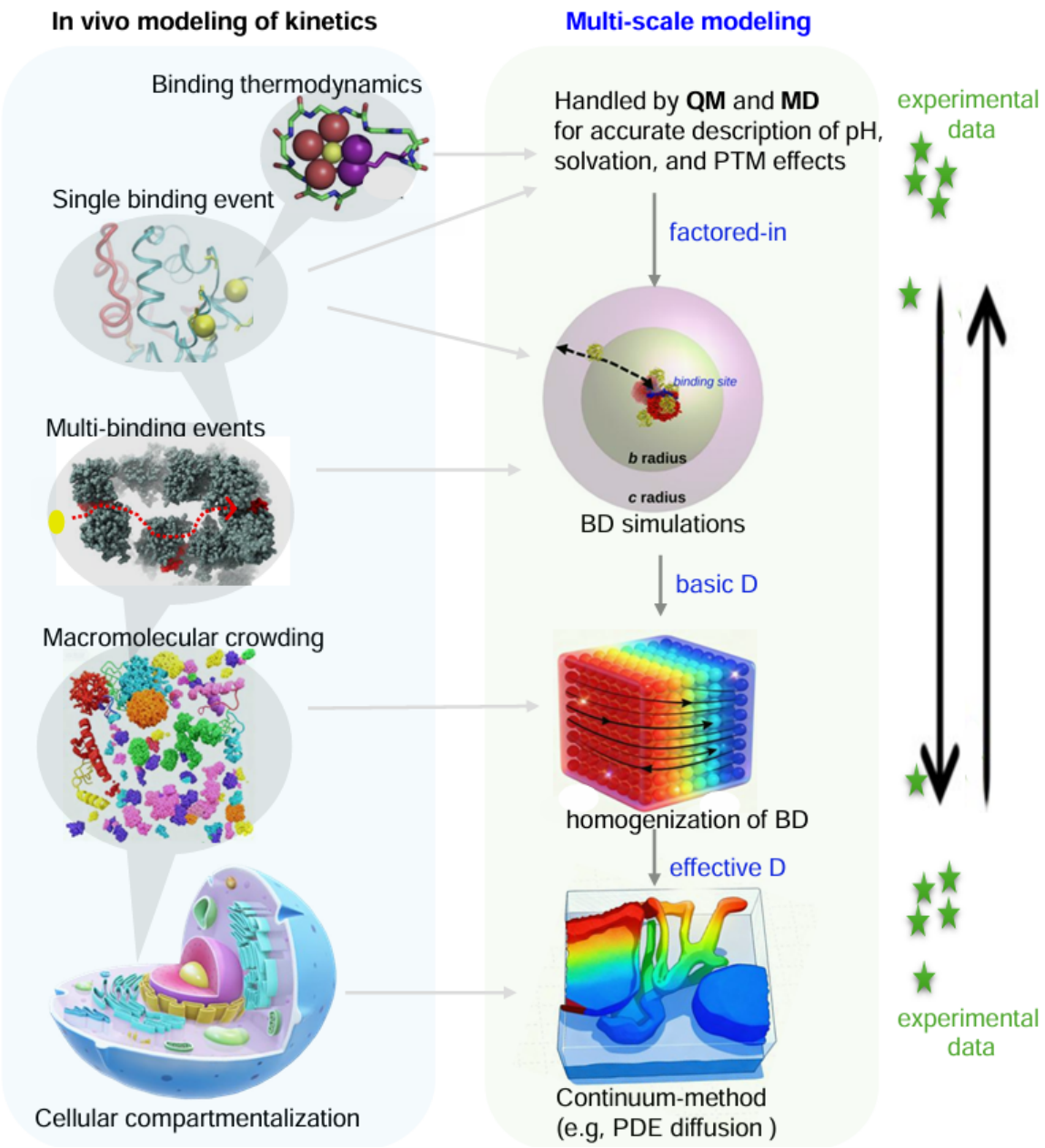

Figure 8: **A bi-directional multi-scale modeling framework for predicting in vivo kinetics.** In vivo binding is a complex process governed by multi-scale events, including cellular compartmentalization, macromolecular crowding, and coupled reactions like metabolic channeling. Furthermore, binding kinetics are modulated by local physicochemical factors such as ionic strength, pH, and solvation effects. To achieve comprehensive insights, a multi-scale computational framework is essential. We propose that such a framework can be formed by integrating existing computational methods across different scales, centered around Brownian Dynamics (BD). The BD technique occupies a central position in the computational tool spectrum: at one extreme, QM/MM provides atomic-level detail but is limited to small scales; at the other, continuum models simulate cellular-level effective diffusion phenomena. Thus, BD can utilize QM/MM outputs for refined diffusion coefficient estimation, while BD results can serve as input for homogenization and continuum methods. Finally, we proposed that such multi-scale modeling framework should be bi-directional. This is because computational insights require experimental calibration, and because experimental data itself spans multiple scales, the entire framework must operate bi-directionally (e.g., bottom-up and top-down) to ensure consistency and utility. .

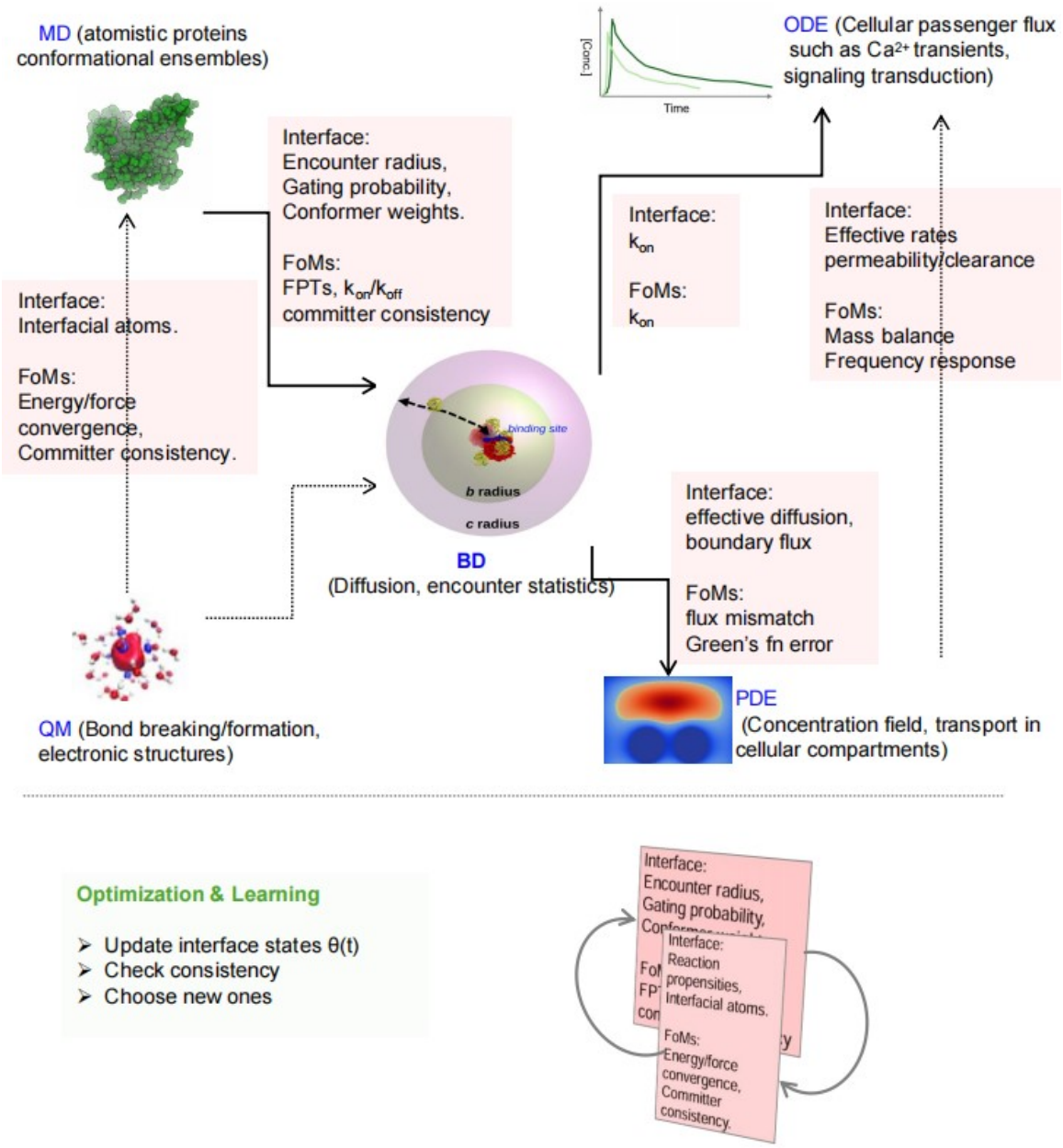

Figure 9: **Multiscale coupling of biomolecular binding models.** Schematic of a bidirectional modeling framework linking quantum mechanics (QM), molecular dynamics (MD), Brownian dynamics (BD), and continuum descriptions (PDE/ODE) of cellular transport and signaling. BD occupies a central mesoscopic role, translating atomistic structure and energetics (from QM/MD) into effective kinetic parameters (k_{on}, k_{off}) and fluxes that inform continuum transport and cellular response models. Interface variables and figures of merit (FoMs) at each scale enable consistency checks and provide targets for optimization, supporting a closed-loop, multiscale learning framework for predicting and controlling biomolecular binding kinetics in vivo..

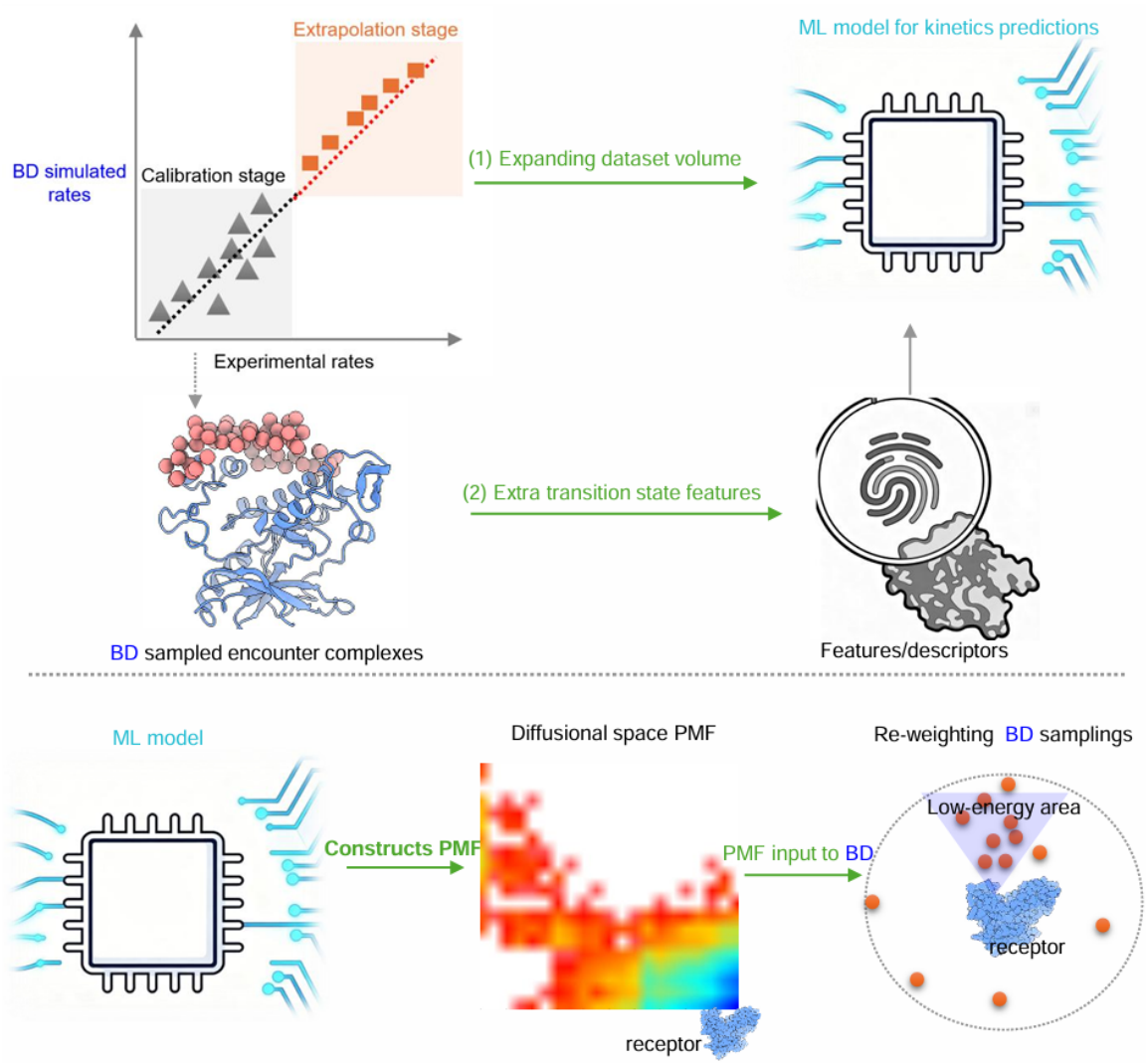

Figure 10: **Using BD and ML for Kinetic Predictions** (A) BD informs ML training. After calibration against experimental data, BD can generate large-scale kinetic datasets for a target receptor, providing the extensive data needed for robust ML model training. Furthermore, BD-sampled encounter complexes naturally encode path-dependent features essential for kinetic prediction, which are often elusive in static, bound-state complex structures. (B) ML enhances BD sampling. ML models can be used to generate potential mean force (PMF) maps, such as those within the diffusional space surrounding a receptor. These ML-derived PMFs can serve as inputs for BD simulations, significantly accelerating sampling efficiency. This approach enables BD to model more realistic, atomically detailed biological binding events, thereby alleviating the inherent sampling burden required for accurate kinetic predictions.. .

Supplement

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