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 multi-scale 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 governed by the speed at which a substrate diffuses to a protein target[1, 2]. Many of these cellular responses result from the concerted 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 discuss later, Brownian dynamics (BD) simulations offer a powerful framework for probing such mechanisms, providing an essential bridge between descriptions of isolated binding events and the concerted reaction schemes to which they belong.

Figure 1: Multi-step reactions. (A) Sequential reactions or multiple binding events are of great biological relevance. For example, the sequential conversion of ATP (or ADP) to AMP and back to ATP, catalyzed by membrane-embedded CD39 and CD73 enzymes, plays an important role in synapse signaling [3] as well as in cancer immunosuppression [4]. (B) Dissection of a basic single binding event into 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 stage, where conformational 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.

1.2 Single Binding Events

A single ligand/protein binding event serves as 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 [5, 6]: 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 their final bound states (Fig. 1B, pink). The "transient encounter" is a non-local process governed by long-range diffusion and electrostatics, whereas 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 for understanding binding mechanisms and their behavior in the cell cytoplasm [7]. From the standpoint of rapid reactions, this requires considering both the rate of formation for 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 [8, 9] 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 [10]. However, many biological binding events deviate from simple spherical models [11]. For instance, the diffusion of quinone within the mitochondrial membrane to shuttle electrons between respiratory complexes [12, 13] (Fig. 2A) would be amenable to a 2D diffusion model, for which analytically solvable models are possible [14]. Additional examples that deviate from the spherical paradigm but permit simple quantitative models include the association of a tethered substrate with its target [15], such as the LIM domains within disordered proteins binding to targets on muscle filaments [16](Fig. 2B), or the association of a

particle undergoing free diffusion in 3D colliding with a surface [17], such as for an extracellular ligand activating a membrane-bound GPCR [18](Fig. 2C).

One-dimensional diffusion-controlled binding events are also possible [19], such as the search a transcription factor performs while sliding along DNA to locate its specific promoter sequence, or binding events involving an ensemble of intermediate states [20]. Although simplified analytic or numerical models can be applied to these cases, atomistic detail or nontrivial interaction potentials often render such approaches infeasible. Here, Brownian dynamics simulations serve as a complementary tool for capturing atomistic detail and nontrivial substrate/target interactions to predict binding kinetics.

Figure 2: Complex environments of binding events, scales, and feedback. Although conceptual binding events often involve only two partners, these events are much more complex in biological systems due to their specialized environments. (A) Substrate diffusion in a special medium such as a membrane; shown are the quinone molecules that diffuse within the mitochondria inner membrane and serve as charge carriers to transfer electrons among respiratory complexes[12, 13]. (B) 'Tethered' actin-binding LIM domain protein (ABLIM) (@2025 Sun et al. originally published in [16]). (C) Agonists from the extracellular environment bind to the cell-membrane embedded GPCR receptors, ultimately promoting 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 EF-hands [21]. (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 [22]. (F) Scaffolded binding of TnI-TnC-TnI that is regulated by Ca^{2+} ions [23]. (G) Oscillatory, negative feedback shown is phospholipase C (PLC), protein kinase C (PKC), and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) cycle adapted from [24].

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 [25], as well as potential contributions from desolvation of hydrated reactants and even quantum chemical effects like polarization and covalent binding [26]. Short-range forces governing association can impact the conformation ensemble by determining the geometric configurations and electrostatic interactions that guide 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 [27–29]. The diverse mechanisms by which a transiently-bound substrate reaches its post-encounter state can dramatically influence the respective binding rates [30, 31]. Since the focus of this review is on forming the transient-encounter complex via diffusion, we refer the reader to robust discussions on this topic [30, 32–34].

1.3 Multiple Binding Events

Binding events in biological systems often feature complexities such as the involvement of multiple reactants, binding sites, or feedback mechanisms. Allostery and cooperativity (Fig. 2D-E) are two examples of these complexities. 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 [35]. Such allosteric regulation is often seen in the GPCR family, where an agonist's binding to the cell-surface receptors induces hetero-trimeric G protein binding on the intracellular side, a distance of approximately 40 Å away [36]. Cooperativity refers to when the binding of one substrate molecule facilitates or prevents the binding of a subsequent substrate of the same type [37]. 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 [21] (Fig. 2D). Another example includes substrate channeling (Fig. 2E), whereby intermediates are guided along enzyme-controlled pathways, often through the spatial co-localization of enzymes [22].

In vitro reconstitution has demonstrated how transient complexes, such as the human 8-oxoguanine glycosylase 1 (OGG1) and human AP endonuclease 1 (APE1) complex, form to facilitate consecutive steps in base excision repair [38]. 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 [23] 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 [4] (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) [39].

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 provide 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 [40, 41]. 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 [42]. 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 [43]. This enhanced binding impairs the heart's ability to relax, a condition 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 [44]. 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 [45].

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 fundamental 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 apo enzyme 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.

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, rather than 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. At a physical level, the association rate (k_{on}) dictates the arrival at the local energy minimum representing the encounter complex, while the intrinsic rate (k_{int}) dictates the subsequent crossing of the main activation barrier to reach the final bound state. (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.

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 [46]. 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 [47]. 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 [48]. 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 highlights the importance of local concentrations of the ligand [A] and target [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 systems out of equilibrium, it is often practical to track the time-dependent concentration changes of the reactants. For a system such as the one described in Eq. 1, the following reaction scheme applies:

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

where k_b represents the dissociation rate, and 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 (k_{on}) and a rate for forming the final complex (k_{int}) (see Fig. 3top). The overall forward rate constant (k_f) incorporates the rates for both the diffusional encounter (k_{on}) and any subsequent intrinsic step (k_{int}), as given by [49, 50]:

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

where k_{on} represents the diffusive encounter rate and k_{int} represents 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 [50].

2.2.1 Factors governing k_{on}: Diffusion-Limited

Many biological reactions are limited not by the chemical step, but by the rate of diffusional search. For these cases, k_{on} reflects the solute's traversal of a potential energy landscape to form an encounter complex (see Fig. 3). In the diffusion-limited regime, these rates typically range from 10^5 to 10^9 $M^{-1}s^{-1}$ [51], compared to intrinsic catalytic rates (k_{int}) that often vary from milliseconds to seconds [52].

Physical factors across a hierarchy of spatial scales, from long-range diffusion to local interactions, collectively shape substrate/target association. At large distances, the diffusion coefficient (D) is a primary governing factor, 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 k_{on} relative to cases where these interactions are negligible [53].. 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 alone [54].

In parallel, long-range mean-field electrostatic interactions can steer molecules toward one another, often increasing rates by orders of magnitude [51]. The barnase-barstar interaction [55] is a classic example, where electrostatic steering drives binding rates to 10^8–10^9 $M^{-1}s^{-1}$ [55]. 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 [56]. Following this initial approach, the system transitions toward a more tightly associated state where short-range forces become dominant (Fig. 4, bottom).

Figure 4: Two-stage ligand/protein binding. 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. An example of gating is seen in inhibitor-HSP90 binding [57], where the binding site's accessibility to ligands is controlled by the conformational states of a lid that transitions between a loop and an α-helix [57]. The refolding of a transient complex into the final bound state can be seen in p53-ASSP protein-protein binding, where simulation-captured encounter complexes exhibited structural fluctuations relative to their initial contact poses, providing evidence of refolding [58]. Coupled binding and folding is a paradigm of the recognition mechanism between an intrinsically disordered protein (IDP) and its globular target, in which the IDP gains secondary structure upon forming the final bound state [59]. Binding can also take the form of fuzzy complexes, typically seen in IDP-IDP binding, such as the H1–ProTα complex [60], where conformational entropy plays a key role in determining the binding free energy. Modern structural biology and AI offer compelling possibilities for probing transient states. For instance, preliminary models of encounter geometries can be generated from programs like AlphaFold-Multimer [61], and 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 states.

2.2.2 Factors governing association kinetics

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. These encompass the events that occur between the transient encounter of a substrate with its target and the substrate's eventual recognition at the binding site. [20] Eq. 6 quantifies how post-encounter processes influence k_{int} in the expression for the forward rate. An important consideration linked to post-encounter events is the energetic cost of conformational adjustments that the protein and its substrate must undergo to achieve the final bound state [62]. Some of these contributions stem from local interactions that provide enthalpic stabilization. Desolvation energy, which is the energetic cost of removing water molecules from the interacting surfaces of the binding domain, is also a critical factor. This process is closely linked to dewetting phenomena, in which transient transitions between wet and dry interfaces influence binding kinetics [63], as well as to "dangling" hydrogen bonds at hydrophobic solute–water interfaces [64] , 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 the ligand and target to their bound conformations [65].

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.[45]. In fact, in vivo drug efficacy often correlates more strongly with the dissociation rate (k_{off}) [66]. 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. This barrier's height is shaped by physical factors, such as the energetic cost of breaking strong short-range non-bonded interactions and the entropic penalty of solvent reorganization during dissociation. 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 associated 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 survey of equilibrium and kinetic assays is beyond the scope of this review, we will highlight several key methods and defer the reader to excellent reviews on these topics and best practices [67, 68]

3.1.1 Isothermal Titration Calorimetry (ITC)

Among the core experimental techniques used to quantify binding thermodynamics like ΔG and ΔH, isothermal titration calorimetry (ITC) is the gold standard [69].

ITC directly measures the heat released or absorbed upon binding [69], through incremental additions of ligand. Hence, ITC is an ideal tool for capturing the thermodynamics of binding resulting from the formation of the post-encounter state. ITC can also be applied to allosteric and cooperative binding events to help decipher the underlying mechanisms. 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 [70]. For such systems, the variable-c ITC strategy, whereby repeated titrations of ligand across a range of protein concentrations may be advantageous. This approach was utilized by Bonin et al. [71], for example, to quantify cooperative binding of dUMP to thymidylate synthase (TS).

In addition to ITC, fluorescence-based techniques discussed later in Sect. 3.2.2.1 can also be used to infer cooperative and allosteric binding mechanisms by monitoring titration curves [72, 73]. 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 [74].

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 [75]. Early quantitative measurements of biochemical reactions often focused on enzyme kinetics [76]. Much of this work was performed using spectrophotometers, which measure reaction rates by monitoring changes in absorbance. These methods are limited to tracking relatively slow processes, making them most appropriate for characterizing steady-state or reaction-limited kinetics [76]. 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 [77], 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 [78], but it is generally not particularly rapid.

3.2 Techniques for Rapid Kinetics and Conformational Dynamics

3.2.1 Fluid-Phase and Surface-Based Kinetics

3.2.1.0.1 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 [79], and it

is well-suited for measuring very fast reaction rates [80]. One of the earliest stopped-flow apparatuses was developed by Chance and Gibson [81]. Since then, the technique has been used in a variety of applications, including capturing fast pre-steady-state kinetics and resolving transient intermediates [82]. In addition, Surface Plasmon Resonance (SPR) is often used for kinetics (k_{on}, k_{off}), including those influenced by diffusion [83].

Label-free techniques like SPR and BLI are widely used for the real-time study of biomolecular interactions and can be used to extract kinetic parameters.

These methods immobilize one binding partner on a sensor surface while flowing 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 [84]; in the dissociation phase, where the bound ligand dissociates from the immobilized chip, the decayed SPR signal is indicative of the dissociation rate [85].In contrast, BLI measures the interference pattern of light reflected from two surfaces [86], with the shift being proportional to the number of bound molecules. BLI is less sensitive to bulk refractive index changes compared to SPR [86]. 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 [86]. Kinetic parameters such as k_{on}, k_{off}, and the equilibrium dissociation constant (K_D) are extracted by fitting kinetic models to these phases.

3.2.2 Structural Dynamics and Label-Based Approaches

3.2.2.1 Fluorescence-based methods The discovery of Green Fluorescent Protein (GFP) greatly advanced cell biology, enabling the observation of molecular processes directly within living cells [87]. 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), for example, fluorescent proteins provide a mechanism to measure time-dependent changes in the distances between interacting probe molecules [88].

Live-cell imaging with FRET provides dynamic, real-time information on interaction kinetics and is enabled by tagging proteins with a FRET pair. Fluorescent probes are typically introduced by genetically tagging interacting proteins with a FRET pair (a donor and an acceptor) to monitor their association over time [89]. Overall, live-cell imaging with FRET allows for the real-time study of protein interactions in the 1-10 nm range [88]. As a result, FRET can provide dynamic and often quantitative information on interaction kinetics [88] and associated conformational changes. FRET-based sensors can be instrumental for monitoring in vivo signaling events, ranging from conformational changes induced by PTMs or substrate binding, to the co-association of components into 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 [90]. As an example, high-throughput kinetic probe competition assays based on FRET have been used to survey drug kinetics across broad classes of protein kinases [91]. In addition, 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 undergoing binding events [73]. Owing to its single-molecule resolution, smFRET can also reveal conformational changes that occur upon association [92]. smFRET-based kinetic measurements have been successfully applied, for example, to study complexin binding to the SNARE complex [93].

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 [94]. This GFP labeling strategy for studying protein dynamics within condensates has been broadly applied, including in bacterial cells [95] and in nuclei where transcription factors form condensates [96]. 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 [97]. 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) [94].

3.2.2.2 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 [98]. With this capability, NMR yields information on molecular structure, dynamics, and kinetics at atomic resolution [99]. Bond rotations on the ps–ns scale can be estimated via relaxation measurements. Relaxation dispersion techniques characterize conformational exchange on the μs–ms timescale [98], while two-dimensional Exchange Spectroscopy (2D EXSY) experiments capture slower exchange rates, such as dissociation processes occurring on the order of seconds [98]. Altogether, NMR is a powerful tool for dissecting complex kinetic mechanisms, including conformational gating [98], 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 [100]. It is worth noting that Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) often complements NMR in mapping allosteric conformational changes and distal coupling upon binding [101].

NMR spectroscopy can also be used to measure binding rates by tracking changes in residue-specific relaxation rates before and after ligand binding [102]. A key advantage of NMR-based methods is their ability to characterize weak binding events, which typically exhibit fast dissociation rates [102]. 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 [103, 104].

3.3 Structure determination

Although not strictly requisite for conducting kinetic and thermodynamic assays, structural data are often instrumental for interpreting results and devising new hypotheses. Specifically, atomic-resolution structures of enzymes provide critical insights into their mechanisms of action. Classically, X-ray crystallography has been the standard for structural interpretation of binding data, such as revealing the cooperative binding between transcription factors and DNA [105]. More recently, NMR techniques and cryo-EM have emerged as strong complements to X-ray crystallography. Ab initio structure prediction is also becoming increasingly popular using techniques such as AlphaFold and RoseTTAFold [61, 106]. A survey of experimental and ab initio structure determination methods provides an ample overview of the state of the art in this rapidly evolving area [107, 108].

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. 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, in contrast to the explicit solvent models typically required for all-atom simulations. This efficiency makes it practical to simulate thousands of trajectories (Fig. 1B), enabling the robust statistical sampling required to study 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 predicting the ensembles 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 the utility of BD, it is helpful to first establish its fundamental physical basis, and 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 the 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, $\mathbf{F}(\mathbf{r})$, 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 out degrees of freedom orthogonal to the reaction coordinate, including the solvent degrees of freedom [46]. 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 over-damped 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 (and thereby diffusion) coefficient fundamentally arises from the fluctuation-dissipation theorem (Eq. 10), which relates the random kicks the solvent imparts on the solute to the macroscopic frictional drag it experiences. 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 the surroundings, resulting in energy dissipation via friction. These concurrent processes are formally linked to the diffusion coefficient by the fluctuation-dissipation theorem. To demonstrate this, we can refer to a Green-Kubo relation [109] that extracts the friction coefficient from the time integral of the random force autocorrelation function:

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

where $\mathbf{F}_{rand}(t)$ corresponds to the instantaneous random force, parameterized as $\sigma\mathbf{N}(t)$ in Eq. 7 [109]. 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 heterogeneous lipid membranes. Beyond dissipation through random solvent collisions, the translational and rotational diffusion of larger solutes can present significant intramolecular hydrodynamic interactions. To characterize these, oftentimes autocorrelation functions of a solute's position or velocity are used in lieu of Eq. 10 [110, 111]:

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

where v_i signifies the velocity of particle i at time t' or 0 and N is the number of particles. Ermak and McCammon provided an extended form of Eq. 8 to capture translational and rotational degrees of freedom, inclusive of hydrodynamic interactions [112], using coefficients following Rotne-Prager [113].

4.2 Transient encounter: BD for Homogeneous Media

4.2.1 Modeling Rigid Solutes

Early treatments of enzyme kinetics involved model systems (often spherical), for which rates were predominantly determined by a substrate's diffusion to a reactive site. We provide an overview of some 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, serving as practical tools for analyzing molecular simulation data and estimating kinetic information.

4.2.1.1 Kinetic models

4.2.1.1.1 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 is a starting point from which refinements can be developed to account for more realistic protein shapes, electrostatic interactions, and reactive surface patches. For instance, the Debye-Smoluchowski model [114] describes a particle diffusing toward and reacting with a spherical enzyme governed by a centrosymmetric interaction potential. This is usually framed (in steady state) as a Laplace problem of the form:

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

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

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

These equations describe the relationship between a particle reactant's concentration $p(R)$ and its diffusion coefficient $D(R)$ at a given radius R. The system is constrained by two boundaries: Γ_{outer} (the outer boundary) where $p(R) = 1$, and Γ_{enzyme} (the enzyme surface) where $p(R) = 0$. This base association rate can be obtained through analytical or numerical solutions of a reaction-diffusion equation, given suitable boundary conditions:

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

When a potential of mean force is important to the association event, the Smoluchowski equation is often used [115]:

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

where we characterize the particle concentration with respect to position, $\mathbf{r}$, and time, t. D is the diffusion coefficient, $\beta \equiv (k_B T)^{-1}$ is the inverse thermal energy, $U(\mathbf{r})$ is a drift term from the interaction potential of mean force, and $p(\mathbf{r},t)$ is 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 $F(r) = -\nabla U(r)$ can be expressed as a continuum diffusion equation via Eq. 16 under specific assumptions [116].

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 [117]:

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

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 [118] and APBS [119]. To quantify their effect on k_{on}, the Debye model can be used [120], 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 [114, 121]:

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

In this expression, the lower integration limit R represents the collision distance, defined as the radius of closest encounter between the two interacting molecules. More generally, k_{on} can be determined from the total flux to a reactive surface [120]:

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

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 approximated for only idealized geometries, which limits their direct analytical application for arbitrary substrate/target binding events.

4.2.1.2 Brownian dynamics Brownian Dynamics is arguably the first mature computational method well-known for streamlining the calculation of diffusion-limited association kinetics (k_{on}) for two atomistically-detailed proteins [6, 122, 123]. To achieve this, BD 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 [122]. 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 MD 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 [123].

The Northrup-Allison-McCammon (NAM) algorithm [6] 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 the practical calculation of association rates. The simulations follow Langevin-like 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.

Figure 5: Newton-Allison-McCammon simulation framework. The NAM framework [6], implemented in the software Browndye and Browndye2 [122], uses BD to calculate the k_{on}. (A) The b-sphere and c-sphere that mark the starting position of the binders and the boundary where the binder reaches the "escape" boundary. By setting reaction criteria that constrain the ligand to bind to a pre-defined binding site, the effects of conformation selection can be accounted for in the calculated k_{on} via Browndye. (B) Browndye2 utilizes 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.

An early example from Northrup *et al*[124] 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 [124]. This solvent effect helps explain 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 [124]. 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 [125]. More recent BD packages commonly reported in the literature include UHBD[126], SDA7[127], Browndye[122], BD-BOX[128], ReaDDy2[129], GeomBD3[130], as well as hybrids that combine BD and MD (e.g., SEEKR[131]). 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 electrostatic-steered binding rates, while ReaDDy2 is designed for reaction-diffusion networks. An excellent review summarizing these BD-based packages is provided by [32].

While BD is computationally more efficient than MD, it can still be subject to significant expense, especially for large or highly flexible systems. To address this computational cost, variants of the NAM algorithm, such as weighted-ensemble BD [132] and biased-BD [133], 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 to evaluate k_{off}. These cases typically apply to events where dissociation occurs through a 'barrier-less' diffusive escape, such as using BD to simulate microtubule disaggregation [134]. 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.

4.2.1.2.1 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. In TST, as developed by 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 $\Delta G^{\ddagger}$ [135] (see Fig. 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 [136] 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 described here for escape (dissociation) processes, it can also be extended to association phenomena when an effective free-energy barrier governs the encounter [137]. A comprehensive review of this theory is available for a further elaboration of these concepts [136].

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 by Berezhkovskii *et al* for BD simulations involving buried binding sites, whose barrier to entry could be elegantly described with a one-dimensional potential of mean force [138]. Including a local diffusion coefficient along the PMF can further improve the accuracy of these kinetic predictions [139].

4.2.1.2.2 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 coordinate 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 direct 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. Importantly,this provides a framework for the numerical estimation of reaction barriers 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 $q = 0.5$. The reverse process can also be resolved by leveraging a detailed balance criterion, whereby the forward flux ($k_{A\to B}\pi_A$) is equal to the reverse flux at equilibrium [140]:

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

Here (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 [141].

4.2.1.2.3 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 discretizing 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 a lag time 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 assumes 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_i, of $\mathbf{T}(\tau)$, using $k = -\tau^{-1}\ln\lambda_i$. One important quantity 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 absorbing microstate, say P_i [142], 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 fundamental matrix, from which the MFPT from any starting state to the absorbing state can be analytically extracted. When the absorbing state corresponds to the bound configuration, the resulting MFPT can provide the association rate 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, where 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) [143] and related frameworks for dimensionality reduction and deep learning—such as VAMP [144], VAMPnets [145], and TICA [146, 147] 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 [131] (discussed in Sect. 5.3.1.2).

4.2.1.2.4 Survival probability 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, transition probabilities for canonical states, such as open versus closed states can be constructed, for instance, using single channel currents from a patched ion channel [148].

Formally, the survival probability, $s(t)$, reflects the probability that a particle remains in a given state over a given time interval, t. [149]. 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 [149].

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

The survival probability has the advantage of being simple to evaluate from 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 [150].

4.2.1.3 Beyond the spherical cow: Anisotropic Reactivity and Dimensionality

4.2.1.3.1 Effects of Dimensionality (2D vs. 3D) Many biological binding events deviate significantly from simple spherical models [11]. Even 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. For instance, the diffusion of quinone within the mitochondrial membrane to shuttle electrons between respiratory complexes [12, 13] (Fig. 2A) is amenable to a 2D diffusion model, for which analytically solvable models are possible under strict boundary conditions [14]. This concept was elegantly discussed 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 [151]. 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 with receptors and channels [152, 153] (Fig. 2D). 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 [154]. Relatedly, receptor tethering onto or embedding into the plasma membrane is a mechanism for confinement that can enhance diffusion-limited association, as seen with the TNFα ligand and its cognate receptor [155]. 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 [156]. 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 [17] (Fig. 2E). One-dimensional diffusion-controlled binding events are also possible [19], such as the search a transcription factor performs while sliding along DNA to locate its specific promoter sequence, or binding events involving an ensemble of intermediate states [20]. 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 [157]. 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 plays a key role in modulating 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 into a conformational selection regime, an induced fit regime, or a mixture of the two [20]. 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} [51]. These two models describe distinct 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 [158], 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 [159]. 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 [160], while others have reported no significant effect [161]. This contrast highlights the case-dependent and complex mechanisms governing the binding of flexible molecules.

4.2.2.1 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 that explicitly considered the flexibility of the active site to achieve more realistic binding rates [162, 163].

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 [164], 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 [164]. This simplified model was capable of capturing the impact of pocket flexibility on predicted association rates.

4.2.2.1.1 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 [165]. 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 the IDPs agreed well with FRET-measured residue-residue distance data [166]. Another study developed an implicit-solvent force field named COFFDROP for Brownian dynamics simulations of proteins and IDRs [167]. This force field derives non-bonded and bonded CG potentials from all-atom MD simulations of all possible two-residue fragments, ensuring transferability. When coupled with Brownian dynamics, this force field performs well in recapturing the hydrodynamic radii of eight IDRs, including Aβ fragment, ProTα, and α-synuclein [167]. In a related work, internal coordinates and chained particle positions were propagated using Brownian Dynamics [168] 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. A Brownian dynamics model for IDRs based on bead-rod chain dynamics, which includes hydrodynamic interactions and excluded-volume effects, has also been developed [169]. It was used to capture the measured internal dynamics of synthetic $(GS)_n$ IDRs ranging from 11 to 40 residues. All of these approaches provide important representations of a protein's intrinsically disordered conformational ensemble.

4.2.2.2 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 [32]. As discussed in detail by Wang *et al*[170], physics-based methods include brute-force MD, MD with Markov state modeling, and Biased MD (such as LiGaMD [171]), which usually require long simulations [59, 172]. 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 [172] and protein kinases [173] without prior 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 [66]. Representative methods include Ligand GaMD (LiGaMD) [171], random acceleration MD [174], metadynamics [175], and WExplore [176]. 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 [177].

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} [178]. 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 [178]. 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 increasingly feasible [66], thereby obviating the need for 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. The 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 condensates to concentrate reactants and thereby improve reaction efficiency [3] (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*[94] showed that under SG formation stimuli, proteins have a reduced diffusion coefficient (D) compared to normal conditions. Under both conditions, evaluating the mean-squared displacement versus time (MSD $\propto \Delta t$) relationship showed that while proteins exhibit a broad range of motion types (e.g., normal vs. confined diffusion), the predominant motion type remains Brownian [94].

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 [94] or time-dependent changes in droplet volume [179]. (B) Modeling with Brownian motion-induced coalescence (BMC). The BMC model [180] serves as a fundamental framework for explaining LLPS dynamics in living cells, including the formation of nuclear bodies [179, 181] and transcription factor condensates [96]. 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 [182]

4.3.1.1 Crowding Protein diffusion in the cytosol often occurs under crowded conditions, which can lead to reduced diffusion coefficients that are often anomalous [183] 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 [184]. The effects of crowding in cells are complex, as reviewed by Zhou, Rivas, and Minton [185]: 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 [183]. Excluded volume effects can also promote association by restricting the configurational entropy to be gained when reactants dissociate [186]. The net impact of crowding on kinetics is a balance between these competing effects [185].

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 overall diffusion, suppressing both k_{on} and k_{off}[185]. For a thorough discussion of simulation with solutes in crowded environments, we refer the reader to Zhou *et al*[185].

4.3.1.2 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 distinct organelles or phase-separated entities like LLPS droplets; eukaryotic cells use compartments like mitochondria and membraneless germline P granules to localize reactions [187]

(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 [188, 189]. Additional considerations are how the physicochemical properties within the condensates themselves can alter catalysis. For example, changes in solvent viscosity and substrate/active site solvation properties can directly modulate enzyme catalytic rates and specificity [190]. 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 activates their enzymatic activities [191, 192].

4.3.2 Modeling reactions in heterogeneous media

4.3.2.1 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[193], which modulates the diffusivity as a function of free volume fraction, $\phi \in [0, 1]$ e.g.

$$D_{eff} = D_0 \left(\frac{2\phi}{3 - \phi} \right) \tag{31}$$

for spherical inclusions much greater in size than the solute [193]. Additionally, these coefficients can be estimated from explicit MD or BD simulations by computing positional or velocity autocorrelation functions (Eq. 11) for diffusing crowders [110].

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 [194]. 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 [195] 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 [196].

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 [197]. Adapted with permission from [197]. (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 [158]. 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 [158]. (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 [198]. Adapted with permission from [198]. (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 [194]. Adapted with permission from [194].

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 explicitly consider the history of the particle's motion (memory effects)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 [199]. Note that many IDPs coagulate to form LLPS (such as ABLIM [200]), so here Langevin [201] and BD simulations could be used [202].

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. [203, 204] (see Fig. 8). 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, which can be used over the larger domain without having to explicitly model the individual crowders. Previous studies have used this approach to model the impact of atomistic-resolution crowders on continuum-scale diffusion coefficients [205, 206], as well as for the diffusion of ATP based on a near-atomistic-resolution representation of the sarcomere [207, 208], which were later shown to agree well with ATP diffusion measurements in skeletal muscle cells [209].

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 propose that such a 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.

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 [210], can be reflected in an effective diffusion coefficient [205, 211] appropriate for large-scale simulations.

4.3.2.2 LLPS modeling BD can also be used to simulate the co-assembly of solutes into a phase separate from their solvent (Fig. 7). As a recent example, Zhang *et al* [182] 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 dynamically 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) [212]. Consequently, modern frameworks effectively couple BD with MD-derived information to bridge the gap between the encounter complex and the native bound state [32].

4.4.1.1 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*[138] 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*[139]. For kinetic effects like conformational gating, BD is often coupled with Markov State Models (MSMs) [158, 213]. 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 [214]. Applications of these coupled approaches range from early modulations using simple gating factors [215] to complex systems including the gated binding of drugs to HIV-1 protease [158, 216], the conformation-selective binding of intrinsically disordered calcineurin to calmodulin [56], and the peptide association of BRCA1-BRCT [217].

4.4.1.2 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 [131, 218, 219]. 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 advanced in subsequent code releases [131, 219].

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 complexes.

4.5.1 Allostery for a single complex

Once a substrate arrives at the transient-encounter hypersurface, its fate is to either diffuse away or transition to 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, which is reviewed extensively in the literature [220, 221]. 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 active sites need not be distant from one another. These concepts are typically approached from an equilibrium perspective, yet an allosteric change can significantly 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 β-arrestin binding on the intracellular side, a distance tens of Angstroms 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 binding kinetics, particularly the residence time (Eq. 30), is a key determinant of which downstream signaling pathway is activated [222].

Extending on the earlier allosteric example, the pre-assembly of a GPCR with its cognate G protein can affect the affinity for its endogenous ligand [223]. This example illustrates the concept of 'scaffolding', whereby target proteins are components of larger macromolecular assemblies that tune substrate affinities. 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 [224]. 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 precise 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 [225] thereby potentially decreasing the dissociation flux of another component as per Eq. 26.

4.5.1.1 Computational and Analytic Approaches

The Hill equation is an analytic approach used 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 binding sites occupied by ligands, and $[L]$ and K_D are the ligand concentration and dissociation constant, respectively. By fitting the Hill equation to experimentally measured ligand-protein binding curves, the Hill coefficient n can be obtained, representing positive cooperativity when $n > 1$ and negative cooperativity when $n < 1$.

Another popular analytical model for studying cooperativity and allostery is the Monod–Wyman–Changeux (MWC) model [226]. This model assumes that allosteric proteins are oligomers composed of identical subunits, akin to a scaffold, 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 single 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 [227].

Beyond these analytical models, molecular dynamics (MD) simulation is a powerful computational tool for revealing allosteric mechanisms in proteins [228]. The primary 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 communication pathways connecting these multiple sites, thereby revealing hidden intermediate states whose stability is sensitive to ligand binding [228]. However, MD generally falls short of sampling a sufficient number of binding and unbinding events to capture transitions between markedly different conformational states. For this reason, while Brownian dynamics (BD) is not traditionally used for insights beyond the initial diffusional encounter, it holds immediate potential for predicting binding kinetics to hidden allosteric sites that are unveiled by a bound ligand. In this capacity, BD could serve as a cornerstone for estimating the K_D in Eq. 33.

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 typically 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 downstream 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 [229, 230]. Under certain conditions, this network topology can give rise to oscillatory behaviors [231].

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 [232]. 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 [51, 233] is a well-understood example, where an abundance of residues presenting an attractive potential to the substrate helps guide it to the target; this generally increases 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 [234]. While this confinement tends to decrease the apparent k_{on}[3], 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, often coupled with favorable electrostatic interactions with an intermediate, prevents significant diffusion of that intermediate away from the reactive centers [235], thereby offering an advantage in accelerating overall reaction rates [236]. However, the benefit of substrate channeling does not always stem from altering diffusion-limited rates, as catalysis itself may be reaction-limited [237]. In such cases, channeling prevents reactive intermediates from diffusing away to be consumed by competing enzymes [238, 239]. 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 [237], controlling metabolite flux at branch points.

Prime examples of substrate channeling are found throughout cellular metabolism. For 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 [22] (Fig. 2A). Similarly, the mitochondrial electron transport chain is another prime example, where quinone molecules shuttle electrons between large, membrane-embedded complexes [12, 13]. Other examples include DHFR [235] and various glycolysis enzymes [240].

4.5.2.1 Computational Approaches

4.5.2.1.1 Kinetic Monte Carlo and Agent-based models Kinetic Monte Carlo (KMC) is a stochastic simulation method that has been effectively 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 physical track for substrate transfer [241].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 $1/k_{tot}$ [242]. 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 [243]. In a study by Klann et al., the conversion of glucose to lactate through four sequential enzymatic reactions was simulated using an ABM [243]. 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 [243].

4.5.2.1.2 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 occurring over larger spatial scales. 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:

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

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

As with Eq. 16, the $\mathrm{k_{on}}$ 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.

4.5.2.1.3 BD simulations of sequential substrate channeling BD can also be used to study substrate channeling and substrate transfer in parallel with experimental approaches. It can model substrate channeling within dynamic enzyme assemblies by simulating the diffusion of an intermediate product from the active site of one enzyme directly 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) serves as 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 [244], and these rates can be explicitly modeled by BD [198].

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 successfully identified the key charged residues that form the electrostatic channel [235], which was followed by a subsequent study investigating the human isoform using similar methodologies [245]. 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 [240].

4.6 Limitations of BD approaches

Several limitations continue to limit the robust utilization of simulations for predicting binding kinetics. The kinetics of enzymatic processes represent a fundamentally more complex problem than the prediction of static binding poses and equilibrium affinities. Enzymatic reactions that occur in vivo involve multiple substrates, essential cofactors, and a sequence of elementary steps, making it significantly more difficult to measure experimentally and computationally predict overall kinetic fitness [246]. This difficulty is compounded by the computational expense of detailed kinetic simulations, especially when solvents are treated explicitly. In addition to computational expense, accuracy can be limited by the quality of the force field parameters used, particularly for transition 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. Implicit solvent models fail to capture the discrete hydration shells and water-mediated hydrogen bond networks that are often critical for the final stages of molecular recognition and binding pocket desolvation[247]. Therefore, a key challenge is the difficulty of creating accurate implicit solvent models that can capture long spatial and temporal correlations. This could benefit from novel network architectures, such as graph neural networks to learn these long-range correlations from explicit solvent data [248].

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 level of 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 these disparate scales (Fig. 8).

We proceed by outlining several research areas essential for modeling the in vivo cellular environment, beginning with processes that involve sequential binding events (Sect. 5.1.1). In Fig. 8, we illustrate the complex factors, from individual molecule encounters to macroscopic 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 and accurate picture. Further, by integrating multi-event modeling with dynamic experimental data, emergent biological insights specific to living systems can be achieved.

The complex binding events occurring 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 such as 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 functional domains. The reduced effective volume, due to crowding, environmental perturbations (e.g., material flux, 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 highly effective for capturing these phenomena.

These three scales are integrated in Sect. 5.3 (Fig. 9), where we discuss multiscale modeling as a necessary 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 critical areas like drug design (Sect. 5.5).

5.1.1 The challenge of in vivo: cellular environments

Enzyme kinetics are frequently studied in isolated, well-controlled in vitro systems. Similarly, most current computational applications reflect simplified conditions in which targets and substrates exist agnostic to their 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 paramount. These conditions inevitably constrain proteins within complexes, leading to significant differences in kinetic outcomes compared to dilute solutions. 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 [249]. Further influencing these factors are covalent, post-translational modifications like oxidation, protonation, and phosphorylation, which can strongly affect the stability of the post-encounter state [237]. Accounting for these complex effects in vivo is challenging, limiting insight into enzyme function within the cellular environment. Starting with the highly dynamic nature of the cellular environment, it is well established that transient changes in pH, protein phosphorylation, and intracellular ion concentrations (such as K^+) can significantly influence enzyme activity in living cells; yet, these dynamic factors are rarely incorporated into standard simulation approaches. We discuss these factors and the role that BD could play in accommodating their impacts on enzyme kinetics.

5.1.2 The Need for Bidirectional, Self-Consistent Multiscale Models

A powerful strategy in addressing these challenges of protein kinetics in vivo 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 simple 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 processes, we can begin to understand how small perturbations within one scale propagate to higher scales and identify emergent behaviors, such as metabolic oscillations [250]. This approach transforms the challenge into accurately defining the important scales relevant to a given in vivo system and establishing robust figures of merit to link and optimize those scales. We propose that BD can play a central, bridging role in advancing these efforts (Fig. 9).

5.2 In vivo modeling in stages

5.2.1 Transient encounter in vivo

5.2.1.1 Cellular changes impacting the transient encounter Brownian Dynamics (BD) simulations are often performed for single-solute systems where macroscopic factors like ionic strength and pH are held constant. However, it is crucial to also explore the cellular context to understand how dynamic cellular states (e.g., fluctuating ATP levels or cell polarization) influence kinetic events in more physiologically-relevant configurations. Several physicochemical factors can continuously 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 reactants are concentrated by translocation to specific cellular compartments or when incorporated into dense lipid rafts. On a similar note, changes in the local environment, such as the formation of lipid rafts or plasma membrane LLPS regions, can alter binding thermodynamics by localizing substrates and hindering their dissociation. Such localization effects can greatly accelerate apparent association kinetics.

Electrostatic interactions and conformational changes influence binding kinetics. Comparing Eq. 15 and Eq. 18 reveals that electrostatic attraction can accelerate binding kinetics beyond the basal rate by modifying the reaction potential energy surface (PES) landscape (Fig. 3). For

instance, a pH change altering a protein's protonation state can shift its net charge, thereby modulating the binding of charged ligands through long-range electrostatic interactions. Similarly, covalent post-translational modifications like phosphorylation can increase a protein's net negative charge. 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 [16]. 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 [251]. 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.

The study of diffusion-limited reactions in complex biological environments, particularly those involving tethering, requires a nuanced approach that accounts for physiological accuracy and environmental variables. One example of localization via tethered components includes the diffusion of a disordered region within TnI to bind a hydrophobic cleft unveiled in TnC upon activation with calcium (Fig. 2F) [252]. 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 strongly influence tether 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 (reviewed in [253]). This localization will, at a minimum, induce complex crowding effects on the intrinsic association kinetics [185]. Current frameworks support the simulation of tethered components either explicitly, using flexible linkers bridging domains, or implicitly, by analytically estimating the local concentration of tethered domains via polymer theories [254].

5.2.2 Post-encounter in the context of cell state

Just as the cellular environment influences the initial diffusional encounter, it can also profoundly affect the subsequent transition to the final bound state. Cellular signaling pathways, for instance, can trigger post-translational modifications (PTMs)—such as phosphorylation, acetylation, and glycosylation—that impact the rate and mechanism of bound-state formation, such as by altering a protein's charge distribution, hydrophobicity, or conformational preferences [255, 256]. These shifts reshape the local energy landscape of the encounter, modifying the stability of transient intermediates and transition states associated with the final bound state. In addition, the physical properties of the local environment, like membrane surface charges and spatial constraints in the cell, can impact how long the ligand remains near the protein following the initial encounter [257]. Together, these biochemical and structural influences bias the conformational selection process and alter the rate at which the final bound state forms; hence, they must be taken into account when using BD, especially for transient encounters between diffusing substrates. To reflect post-translational modifications in BD simulations, events such as phosphorylation could be represented as discrete states for a given molecule, though this approach would realistically be limited to a tractable number of PTM combinations.

5.2.3 Multiple binding events in vivo

A holistic view of cell biology entails considering multiple, sequential binding events that may unfold in parallel. This is exemplified in the sarcomere and its target proteins, where the diffusion-influenced binding of calcium, Ca^{2+}, to troponin C is coupled to the kinetics of cross-bridge cycling. This presents two key challenges. The first challenge is to accurately reflect the kinetics of the individual 'reactions' within the cellular environment in isolation, often while constraining their rates based on established data from in vitro or in vivo experiments, or potentially even other well-characterized cellular systems [258]. A second challenge is that most studies only consider a few reactive centers in sufficient explicit detail due to computational or modeling limitations.

From there, a key challenge involves determining free parameters for states of a system that are hidden from direct measurements, such as the gating of ion channels. Often, several model topologies can describe the observed data, requiring the evaluation of different parameter values and combinations to ensure consistency. As a result, these solutions are generally not unique and can permit a wide range of parameter values [259]. A variety of optimization techniques have been actively used for this purpose, including genetic algorithms and Bayesian inference [260, 261]. There are other constraints that may be imposed, such as using the free energy of ATP hydrolysis as a constraint for ATPases [262–264]. 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 [262, 263]. Other constraints may entail ensuring that compartmented or spatially-explicit models yield spatially-averaged values that are temporally consistent with whole-cell measurements [265, 266]. To our knowledge, analogous constraints are generally not imposed on the calculation of diffusion-limited reaction rates.

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 assemblies of oxidative phosphorylation complexes portrayed in Fig. 2A. In this complex, the kinetics of the respective complexes' dynamics, variations in their relative positions, and the electro-diffusion of the many reactants and products create a combinatorial number of configurations, all of which 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 steady state. This approach is similar to how continuum reaction-diffusion models handle multiple species, such as by setting the left-hand side of Eq. 34 to 0. In addition, any biological processes are described as rare events, but can still be parameterized, for instance, by calculating waiting times based on Poisson distributions [267] Even for infrequent events, tens of thousands of these events may occur over a sufficiently large volume, which permits 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 signal 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, the substrates involved can shift dynamically between these phases, for example, through liquid-liquid phase separation (LLPS) or the aggregation of LIM proteins binding to the sarcomere [200]. In some cases, these phases can be modeled as independent, homogeneous systems, but they require careful consideration of the interfacial terms that impact solute partitioning. This is related to multi-phase problems where independent domains are bridged through an interface like a lipid bilayer—for example, the transport of material through aquaporins, VDAC, and nuclear pore complexes. The transduction of an extracellular ligand binding to a GPCR to promote intracellular G-protein activation is another example of information passing between coupled compartments.

5.3 Multiscale modeling of in vivo systems:

In the preceding section, we highlighted a number of complications that arise in the simulations of reactions within in vivo systems. Here we highlight key scales that a typical binding event might entail in vivo, and how linking multiscale models via BD to interpret and predict kinetics affords an attractive solution. At the macroscopic level of cell signaling, continuum models describe species by their average concentrations, either across an entire 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 describes the critical conformational motions of enzymes as they align with substrates. The catalytic conversion itself is a quantum mechanical phenomenon involving the rearrangement of electrons and atomic centers. 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 for modeling biological events. Although robust methods exist for individual scales, developing computationally efficient frameworks to connect them remains a challenge. This process involves defining appropriate fine- and coarse-grained models and establishing rigorous procedures to transfer information between them. The reaction–diffusion equation (Eq. 16) provides an example mathematical basis for connecting these scales. Here, the goal is to show how diffusion ($D\nabla^2 C$) is handled at the continuum level while the PMF reflects energetics estimated from particle-based modeling.

We propose BD as a simulation paradigm uniquely well-suited to enable the 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 [268]. 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 interfaces including BD.

5.3.1 Exchanging microscopic information with BD

5.3.1.1 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 states and transition states at stationary points along a reaction pathway and their corresponding zero-point energies [268]. 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 set of nuclear coordinates, and then the forces acting on the atoms are used to advance the atomic positions in the classical molecular mechanics/dynamics domain until figures of merit like energies and forces converge. Popular tools that use this method include ONIOM [269] and the generalized hybrid orbital (GHO) method [270]. While useful for predicting reaction pathways and providing estimates of reaction rates [271], QM/MM simulations are generally limited to timescales up to tens of picoseconds, significantly shorter than the millisecond scales required for large 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 [272]. 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 [273]. PCM calculations rely on defining a solute/solvent interface as 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 target could provide substrate/target binding poses as starting points for QM treatments. Specifically, these poses would serve as inputs for QM calculations which in turn generate updated electron densities as inputs for the electrostatic potential used for BD. BD serves an important role here by providing rapid sampling of transient-encounter complexes, whose conformations can be refined through more detailed MM and QM protocols. Convergence of the two models could be based on when the BD predicted substrate poses adequately saturate the target surface that is used for the QM based electrostatic potentials.

5.3.1.2 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[274]. Although MD simulations have been used in tandem with Brownian dynamics (BD) in numerous applications, they have conventionally been linked in an ad hoc manner, usually by identifying starting conformations with MD with which rigid-body BD simulations are then performed [56, 275]. The recently proposed SEEKR approach offers a more systematic solution that directly couples BD to MD. [219]. Analogous to the QM/MM example, it is based on defining a boundary surface dividing the two simulation regimes, namely the bound and fully dissociated states. Near the bound configuration, 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 the specific binding mechanism. 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 adaptive boundary could be triggered by assessing the variability of the potential energy surface (defined through parameters like substrate/target distance and orientation) at a given boundary. A smooth potential would be expected where non-bond interactions are weak and appropriate for BD simulations, whereas roughness would signify strong intermolecular interactions amenable to MD. This boundary could be varied iteratively until the k_{on} figure of merit in Eq. 32 reflecting the combined MD/BD contributions achieves convergence between iterations.

5.3.1.3 CGMD:BD Coarse-grained (CG) particle simulation approaches that use reduced representations of atom groups can sample temporal and spatial scales beyond the reach of conventional atomistic simulations. Common strategies include the Martini [276] force field for molecular dynamics and COFFDROP [277] for Brownian dynamics, In these models, groups of atoms are represented by single beads, and force fields are parameterized 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

[278]. Most applications of these CG approaches tend to be unidirectional, in so far as they use atomistic-resolution data strictly as model inputs to capture coarse-grained properties like radii of gyration [278] or aggregation propensity [277].

Efforts to back-map 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*[279] have not been applied to simulations of bi-molecular association. In principle, back-mapping strategies applied to COFFDROP/BD-based simulations like the IDP aggregation events studied by Ahn *et al*[278] could enable the validation of specific interactions at the atomistic level and be used to iteratively 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 the adaptive boundary approach we described. These could be interfaced by a sharp spatial boundary, whereby CGMD yields candidate substrate/target poses, whose transient encounters are modeled via BD. 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 [280].

5.3.2 Exchanging macroscopic information with BD

5.3.2.1 (MD/BD):ODE The examples discussed so far largely 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 significant momentum in recent years, as exemplified by work from Silva *et al*[281]. 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, such as from Guerra *et al*[264]. For instance, Teitgen *et al*[275] combined Brownian dynamics and MD to demonstrate how deoxy-ATP, an ATP analog, impacts myosin/actin association; this was in turn combined with an ODE model to predict cardiac contractility at cell and organelle levels. Relatedly, our study of ABLIM1 [16] 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 simply 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 simulations provide 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 [282]. The 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 [283] 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 [284]. 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 as-

signments [122], are adjusted to reproduce experimentally measured rates. By analogy, one could algorithmically tune the reaction probability or energy thresholds within a BD simulation until the BD-predicted flux matches the macroscopic dA/dt from the ODE. A risk with this strategy is in the case that the ODE parameter is a heuristic from fitting that is not compatible with physical reality, such as an unphysically fast diffusion rate. Here, the BD may provide bounds in that parameter's values, which would serve as constrains for refitting the ODE until consistency is achieved.

5.3.2.2 BD:Continuum Linking particle-based simulations (BD or MD) with continuum representations remains a 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*[282] 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 [208] or reaction rates [285] suitable for continuum modeling. We have successfully applied this framework to estimate D_{eff} and derive rate constants from MD for use in continuum transport problems in materials [205]. Strategies such as parameter lumping can consolidate rate equations for processes that equilibrate rapidly relative to the timescale of the continuum system [263], which can help align catalytic mechanisms with atomistic models [264], while reducing the problem stiffness and ill-conditioning [286] that frequently 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 its 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 alongside Brownian dynamics based simulations of ion movement [287, 288]. Various boundary conditions are applied where the pore intersects with a continuum reservoir of ions to ensure physiological ion gradients are maintained, as summarized in [289]. More advanced hybrid BD/continuum schemes [290] support bidirectional exchange by passing particle positions and continuum concentrations through a shared buffer region, ensuring conservation of both mass and momentum. Successful efforts to bridge BD with continuum models have traditionally focused on passing particle positions and their continuum representations across a shared boundary, as was done by Huber *et al* for charged particles using a 'buffer region' [291]. However, many existing MD–BD–continuum couplings rely strictly on static boundaries. Allowing BD–continuum interfaces to evolve dynamically by adjusting boundary placement or permeability to maintain flux continuity would greatly enhance bidirectional information flow and potentially improve overall computational efficiency.

5.3.2.3 The Feedback Loop: Closing the Circuit with Differentiable Multiscale Modeling As suggested by these examples, a differentiable multiscale modeling framework centered tightly on the BD interface region could provide a unified strategy for linking particle-based and continuum models. The key advantage lies in establishing a true feedback loop that enables information to flow seamlessly in both directions. This would enable the backpropagation of macroscopic observables (e.g., tissue-level dynamics) down to fine-grained molecular parameters, and then propagating those updated microscopic details back to higher-level dynamics. This can be achieved by self-consistently updating shared properties and dependent variables across scales—for example, by enforcing continuity of chemical potential or the strict conservation of particle flux [292]. An error function might take the form $\epsilon \equiv |\lambda(\psi) - \lambda_{\text{exp}}(\psi)|$, where ψ is an optimizable boundary coordinate and $\lambda(\psi)$ is a measured property. 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 the error between predicted and target figures of merit. This approach assumes a smoothly varying error function amenable to optimization strategies like steepest descent, which may be unlikely given the inherent noisiness of simulation data. Techniques such as dimensionality reduction (e.g., identifying reaction coordinates that smoothly represent a process) may be helpful. This facilitates the ability to backpropagate from macroscopic observations down to molecular parameters. For example, the model could automatically adjust parameters at the molecular scale, such as a drug's residence time at a cryptic allosteric site, in order to minimize the difference between the simulated and observed cellular response. Such a framework would provide a systematic, data-driven means to refine molecular parameters to align with desired macroscopic behavior, effectively 'closing the circuit' of multiscale model 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 to succeed. Machine Learning (ML) offers a promising path to scalability by serving as rapid surrogate models for approximating 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[293]
RF	k_{off}	Protein-ligand (406)	X-ray/docking	distance based protein-ligand atom pairs	R^2 = 0.384	2020 [294]
PLS	k_{off}	p38 kinase (32)	X-ray	protein-ligand interaction energies (EvdW Eeel)	R^2=0.79	2021[295]
RF	k_{off}	PDBbind-koff-2020 (680)	X-ray/docking	distance-based protein-ligand atom pairs	R^2 = 0.498	2022 [296]
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[297]
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 [57]
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 [298]
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 [299]

[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

5.4.1 Prior art

Machine learning (ML) has revolutionized the prediction of protein structure, as exemplified by breakthroughs from RoseTTAFold [106] and AlphaFold [300] in generating surprisingly accurate models from sequence alone. This success extends to protein design with diffusion-based models like RFdiffusion [301] and to functional interpretation using advanced protein language models, such as ESM-2 [302]. 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 binding kinetics. Surprisingly, 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 [303], their extension to kinetics is less common.

A survey of ML studies on binding kinetics from 2020 to 2025 yield a small collection of studies. These studies focus almost exclusively on predicting the k_{off} rate for protein-inhibitor binding (see Table 1), with no ML models currently reported for predicting k_{on}. This paucity of kinetic ML models is unsurprising, given the limited volume of experimental kinetic data available. 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 [304]. Its latest update in 2025 incorporated RNA-protein binding kinetics [305]; 2) The *PDBbind-koff-2020* dataset, containing 680 k_{off} data points for protein–ligand complexes [296]; 3) A dataset of k_{on} and k_{off} values for 104 inhibitors targeting HSP90 [212]. 4) A dataset of k_{on} and k_{off} measurements for 270 inhibitors against 40 kinase targets [91]. 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 [150].

Beyond the limited data for training, the bias toward k_{off} prediction is consistent with drug residence time ($1/k_{off}$) as strong predictors of vivo drug efficacy [306]. 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 derived from transient encounter complexes, which are largely elusive.

5.4.2 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 simple state function, making it a data-intensive problem at the molecular level.

Here BD can be used to expand the coverage of kinetic datasets for ML model training. Specifically, BD simulations can be used to generate extensive ligand/protein binding trajectories that can be calibrated against experimental data available (Fig. 10).

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 the 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 of mean force (PMF) maps, such as those within the diffusional space surrounding a receptor. These ML-derived PMFs can serve as direct 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.

The selection of appropriate features or descriptors is critical for the strong 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 unbound/bound states. Therefore, ML features designed to predict these rates must be informed by these states. Most current ML studies on k_{off} prediction use features based heavily on bound-state complexes, such as ligand-atom pair distances [293, 296] or interaction energies [297]. This implies that k_{off} values are largely correlated with K_D and that bound-state features are sufficient. While this assertion is supported by one curated database [293], the authors of a kinome-inhibitor kinetics database claim that k_{on} values actually correlate more strongly with K_D than k_{off} values [91]. This raises the significant question of whether bound-state descriptors alone are truly sufficient. A major challenge is that descriptors often under-represent the role of protein dynamics and additional 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 reduce this limitation by efficiently simulating thousands of substrate-enzyme encounter complexes. Meanwhile, outputs from BD, such as electrostatic potential maps or encounter complex statistics, could serve as valuable input features for ML model training (Fig. 10).

In turn, there are burdgeoning opportunities for ML to directly enhance BD simulations. ML methods can be used to directly improve BD-derived rate estimates or to enhance the underlying simulation data driving them. One critical area is the determination of Potentials of Mean Force (PMFs) describing substrate-target association pathways, estimations which are essential inputs for BD. ML methods are increasingly integrated with PMF techniques to improve the accuracy of those derived from MD simulations [307]. An ML model could learn a more accurate PMF or estimate the diffusion coefficient along a PMF surface, thereby accelerating or improving the baseline accuracy of BD simulations. These refined PMFs can help prioritize exploration of under-sampled regions of diffusional space, greatly enhancing overall BD simulation efficiency (Fig. 10). AI could also be used to optimize reaction coordinates for sampling and reconstructing PMFs in poorly-sampled regions, such as through the use of autoencoders [308] .

5.4.3 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 overall statistical power of models. Moreover, existing kinetic data are often heterogeneous, having been measured under varying experimental conditions, which complicates the curation of consistent and reliable 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 that are absent in unseen targets rather than learning generalizable principles [309]. Consequently, high-quality datasets are essential for implementing homology-aware splitting, where training and testing sets are systemetically divided to ensure no close evolutionary relationship exists between them. This ensures the model ascertains transferable physicochemical rules rather than dataset-specific biases. An open challenge is that a training set enriched in BD data will learn the biases inherent to the BD simulation as opposed to physical reality. Therefore, careful curation of input data sets and continuous retraining as more experimental data are accrued will be necessary to mitigate this risk.

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 benefits from the use of advanced network architectures, such as 3D Convolutional Neural Networks (CNNs), which are 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 driving association [310].

5.5 Applications

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

5.5.1 Drug Design and Systems Pharmacology

In modern drug discovery, binding kinetics are recognized as 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 [311]. 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 overall pharmacological effect in a dynamic, open physiological system [66]. Sustained target inhibition is believed to provide a much more durable therapeutic, making kinetics a key consideration in modern drug development [312]. For example, a long residence time (slow k_{off}rates) can be more important for in vivo efficacy than binding affinity in many kinase inhibitors, such as those targeting the PI3K pathway [311]. Indeed, Georgi *et al* demonstrated that for inhibitors targeting kinases, drug efficacy often correlates with target residence time [91].

Once the cellular state is well-characterized, multiscale modeling can be applied to optimize the design and delivery of therapeutics. Simulations that account for the complex in vivo environment can define critical therapeutic windows, thereby placing explicit constraints on the necessary k_{on} and k_{off} values for a successful drug. Because many drug-target association events are reaction-limited rather than diffusion-limited, the binding process is often governed by conformational selection, induced-fit mechanisms, or rebinding rates [313]. Consequently, computational models offer a powerful framework for designing drugs that target specific, transient functional states, such as intermediate states of ion channels, as opposed to simply optimizing affinity for a single static structure. For instance, the Clancy group has modeled ion channel gating kinetics to identify drugs that specifically target intermediate states of the ryanodine receptor (RyR) to correct arrhythmias [314]. Here, BD is particularly well-suited to resolve the kinetics of diffusion and binding to such transient or cryptic sites, enabling the design of pathway-specific drugs that finely tune residence times and therapeutic efficacy [315]. Building on this, a more sophisticated drug design strategy could involve stabilizing specific functional conformations through allosteric trapping. For example, we recently used Bayesian inference to infer calcium uptake rates by the SERCA pump [259]; this framework could be combined with BD simulations to model how certain endogenous peptide modulators prefer the pump's E1 state while others target its E2 state, thereby directly impacting Ca^{2+} pumping rates.

A differentiable, multiscale approach in this context could go far beyond optimizing a single parameter like affinity by allowing for the simultaneous co-optimization of multiple, interconnected properties under biological constraints. For instance, one could rationally design a drug that not only possesses a long residence time but also exhibits highly favorable transport properties in a crowded domain like the sarcomere, all while reducing off-target interactions.

5.5.2 Peptide design for dynamic processes

A BD-centric framework for integrating molecular-level alterations with cellular-level outcomes could be invaluable for sensitively probing and designing signaling pathways. As an example, a HEK 293 cell line transiently expressing the SERCA pump can be used to characterize the pump's dynamic regulation by membrane-resident peptides like phospholamban (PLB) [316, 317]. 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 computationally 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. BD can bridge atomistic conformational changes to mesoscale concentrations by resolving substrate and target diffusion within crowded cellular environments.

This setup provides an ideal scenario for multiscale approaches to bridge micro and macro scales. The connection demonstrates 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 peptide drugs to 'trap' specific protein conformations, for example, by modulating RyR ion channel gating kinetics [314]. Conversely, a protein's instrinsic 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 synthetase (FAS) [318] and the HSP90 protein [57]. Given that BD's core strength is its ability to bridge atomistic details to mesoscale properties, it not only complements continuum models of diffusion but also enables mechanistic predictions of how local molecular interactions shape cellular signaling outcomes.

5.5.3 Extension to Other Biological Systems

In biological systems, reaction–diffusion processes at the molecular scale often have direct mathematical analogues at longer time and larger spatial scales involving higher-order entities like macromolecular complexes or even cells. For instance, reaction diffusion models have been used to study viral capsid [319] and actin filament assembly [320].In addition, given that isolated cells can exhibit Brownian-like motion and directed migration along chemokine gradients under specific conditions, 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 [321]. This apparent scale invariance suggests that the fundamental concepts of Brownian Dynamics are transferable from molecules to larger assemblies. This correspondence opens new opportunities to apply diffusion-based modeling across multiple scales of biological organization, translating insights from molecular signaling to tissue-level dynamics.

5.5.4 Model validation and experimental alignment

Lastly, the predictive power of computational models is fully realized when rigorously validated and constrained by experimental data. In this regard, there is a critical need for the extensive alignment of experimental and computational datasets. Such alignment priorities were raised in a review of matching molecular crowding experiments with biomolecular simulations [322], but these challenges only grow in complexity when considering dynamic molecular association events. To rigorously guide those alignments, statistical frameworks such as Bayesian inference, maximum entropy, and maximum likelihood estimation serve a prominent role. These approaches enable the integration of diverse datasets, ranging from time-resolved X-ray crystallography and NMR to FRET and stopped-flow kinetics, by refininf simulation parameters until they optimally reproduce experimental observations [323, 324]. For example, a recent study demonstrated how Bayesian inference could be used to parameterize ODE-based systems biology models directly from live-cell fluorescence imaging data [259], suggesting the potential feasibility of using such methods to parameterize simulations like BD. However, the statistical alignment of datasets imposes intense computational demands; consequently, dimensionality reduction techniques such as regularization and projection (reviewed in [325]) could be essential to narrow the parameter space for evaluation .

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, dynamic phase separation, and electrostatic steering do not merely perturb isolated binding events, but reshape the underlying energy landscapes that govern them. Although hybrid BD–MD approaches have emerged as powerful strategies for accurately 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 paradigm shift toward self-consistent, bidirectional, multiscale models. We propose the accelerated development of differentiable frameworks featuring closed feedback loops, in which macroscopic continuum fluxes act to physically constrain microscopic BD transition probabilities. Such a unified approach would allow biological systems to be simulated as deeply coupled, optimizable entities across vast scales, opening powerful new avenues for both understanding and rationally engineering complex biological processes in vivo.

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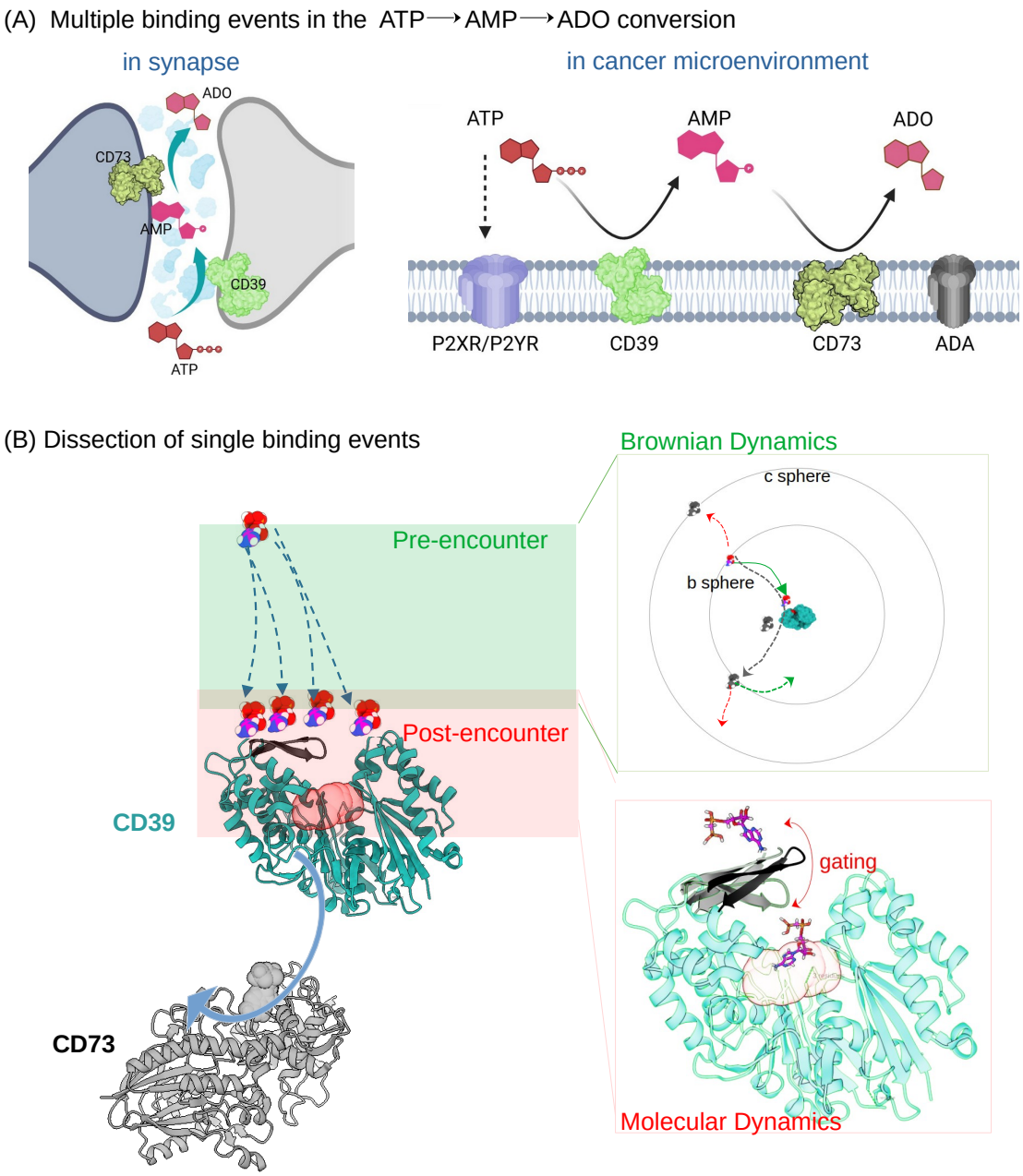

(A) Multiple binding events in the ATP⟶AMP⟶ADO conversion
in synapse
in cancer microenvironment
ADO
CD73
AMP
CD39
ATP
ATP
AMP
ADO
P2XR/P2YR
CD39
CD73
ADA
(B) Dissection of single binding events
Brownian Dynamics
c sphere
b sphere
Pre-encounter
Post-encounter
CD39
CD73
gating
Molecular Dynamics

Figure 1: .

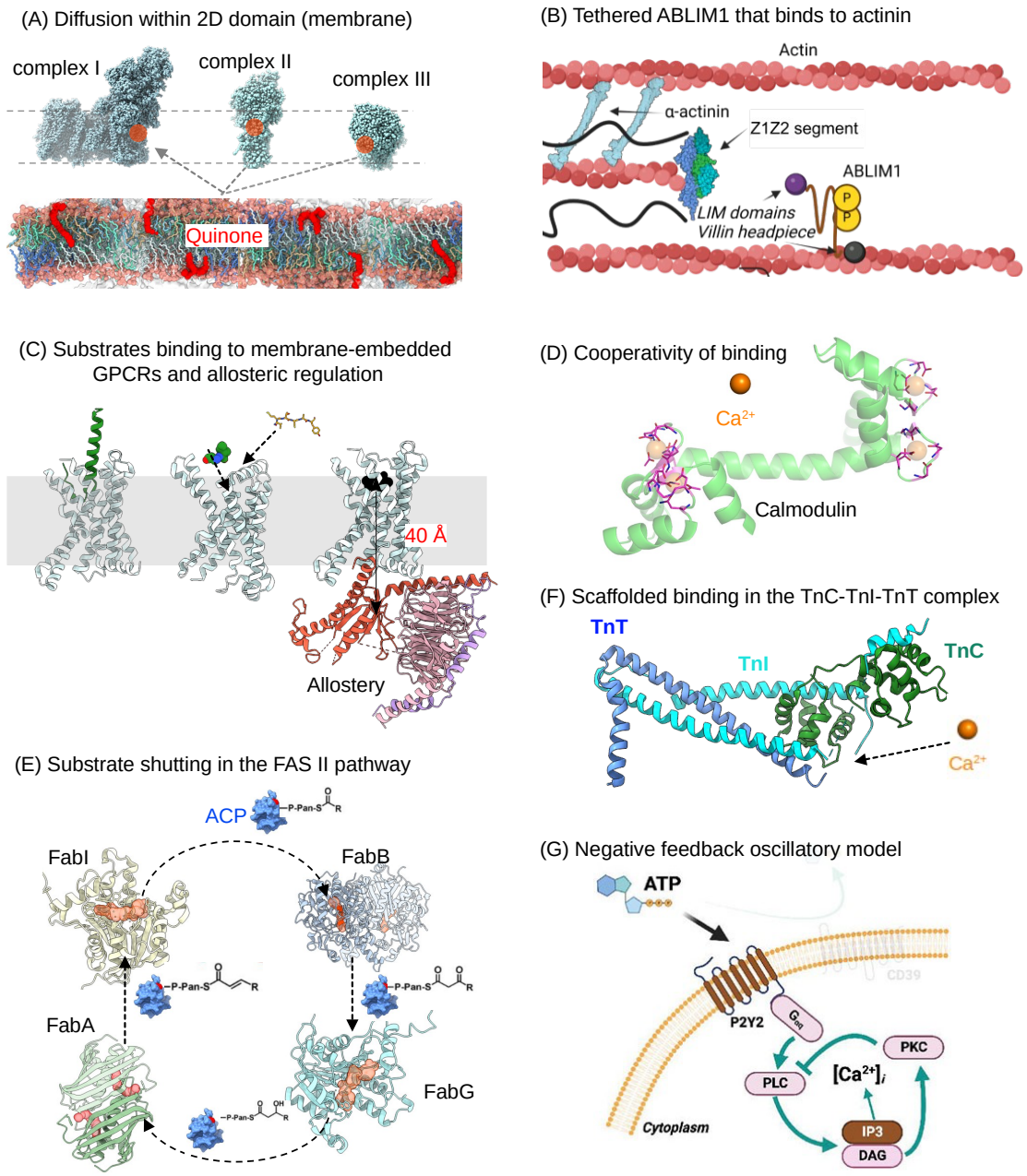

(A) Diffusion within 2D domain (membrane)
complex I
complex II
complex III
Quinone
(B) Tethered ABLIM1 that binds to actinin
Actin
α-actinin
Z1Z2 segment
ABLIM1
LIM domains
Villin headpiece
P
P
(C) Substrates binding to membrane-embedded GPCRs and allosteric regulation
40 Å
Allostery
(D) Cooperativity of binding
Ca2+
Calmodulin
(F) Scaffolded binding in the TnC-TnI-TnT complex
TnT
TnI
TnC
Ca2+
(E) Substrate shutting in the FAS II pathway
ACP
FabI
FabB
FabA
FabG
(G) Negative feedback oscillatory model
ATP
CD39
P2Y2
Gαq
PKC
PLC
[Ca2+]i
IP3
DAG
Cytoplasm

Figure 2: .

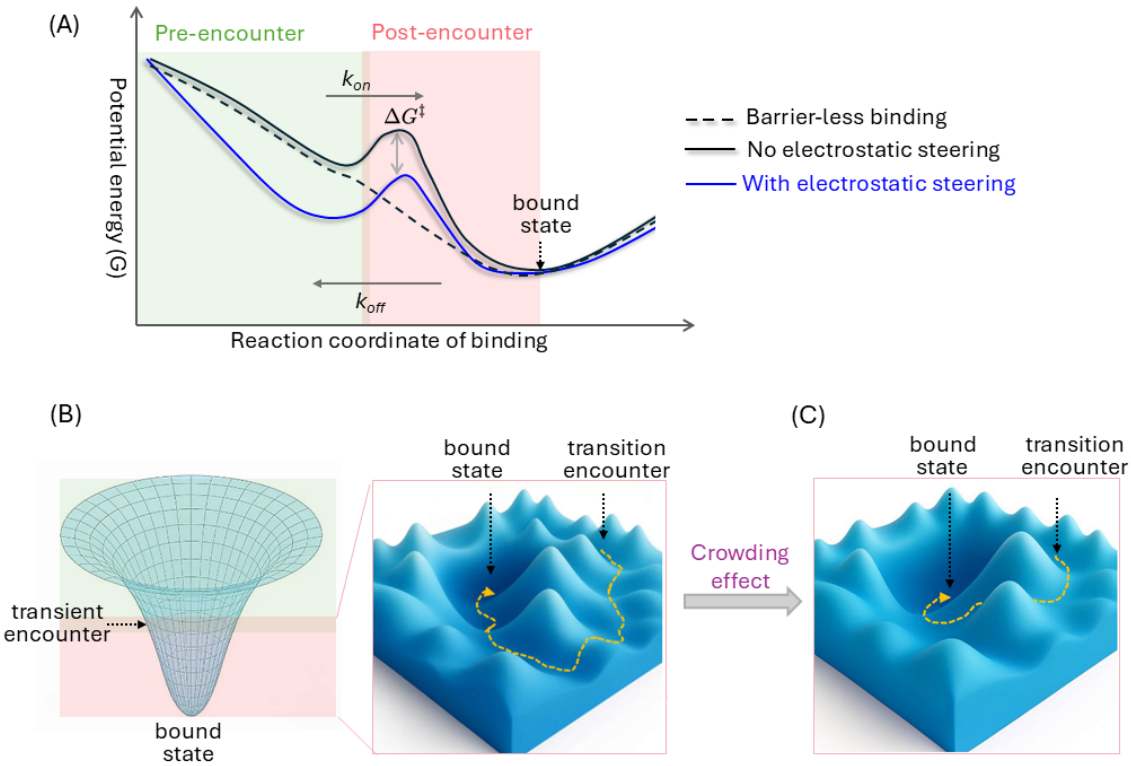

(A)
Pre-encounter
Post-encounter
k_{on}
$\Delta G^{\ddagger}$
bound state
k_{off}
Potential energy (G)
Reaction coordinate of binding
Barrier-less binding
No electrostatic steering
With electrostatic steering
(B)
transient encounter
bound state
bound state
transition encounter
Crowding effect
(C)
bound state
transition encounter

Figure 3: .

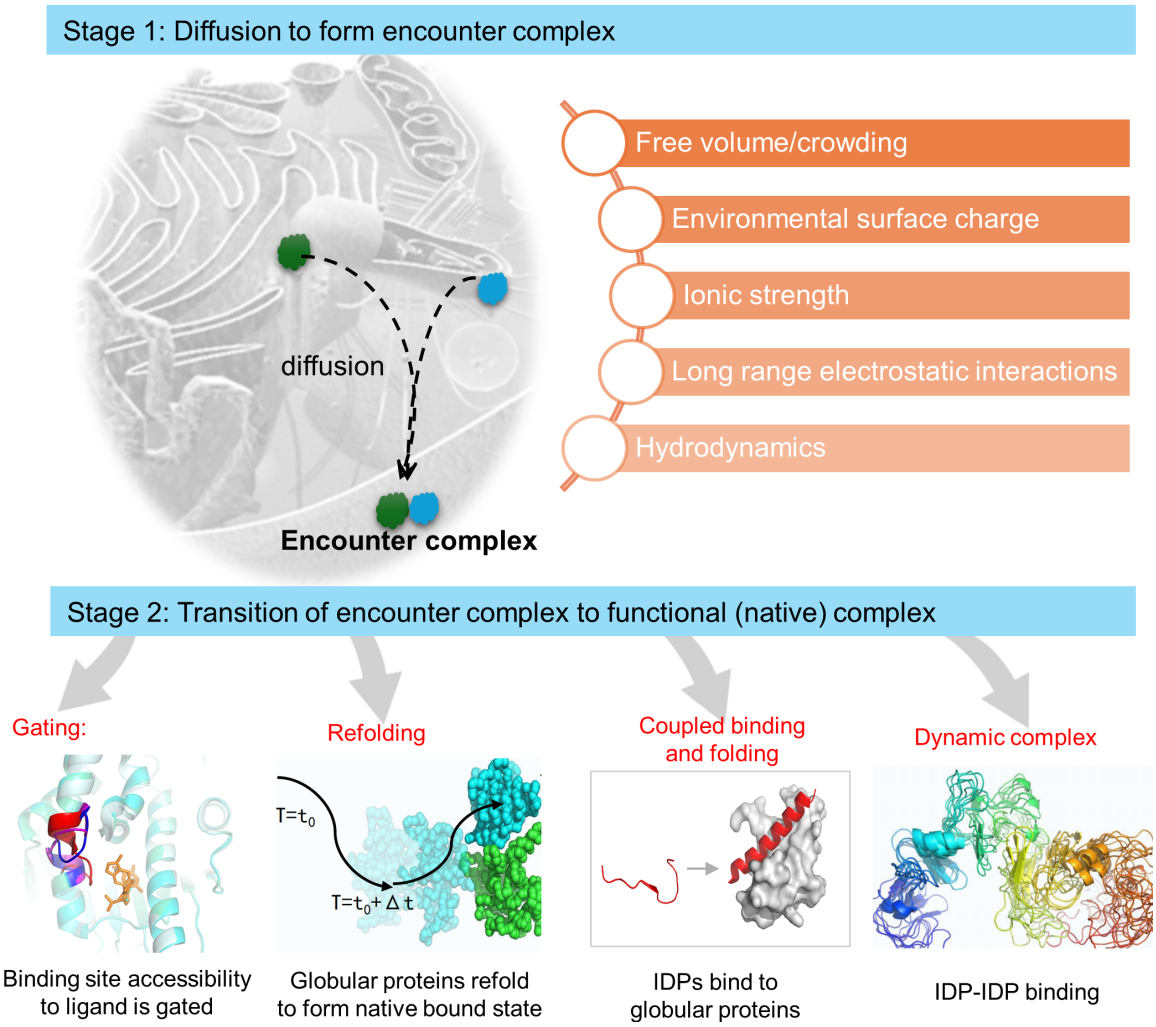

Stage 1: Diffusion to form encounter complex
diffusion
Encounter complex
Free volume/crowding
Environmental surface charge
Ionic strength
Long range electrostatic interactions
Hydrodynamics
Stage 2: Transition of encounter complex to functional (native) complex
Gating:
Refolding
Coupled binding and folding
Dynamic complex
T=t0
T=t0+Δt
Binding site accessibility to ligand is gated
Globular proteins refold to form native bound state
IDPs bind to globular proteins
IDP-IDP binding

Figure 4: .

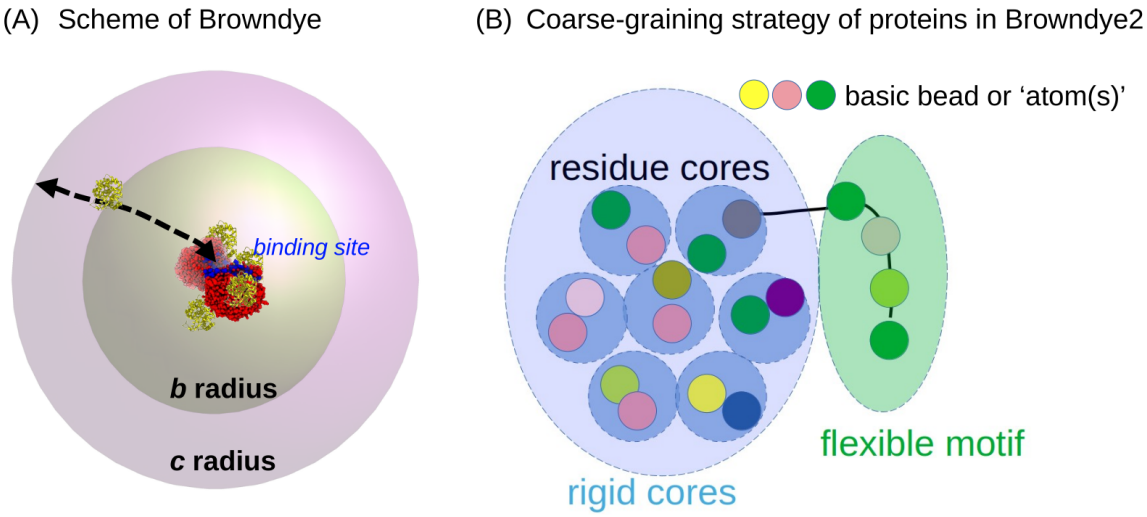

(A) Scheme of Browndye
binding site
b radius
c radius
(B) Coarse-graining strategy of proteins in Browndye2
basic bead or 'atom(s)'
residue cores
flexible motif
rigid cores

Figure 5: .

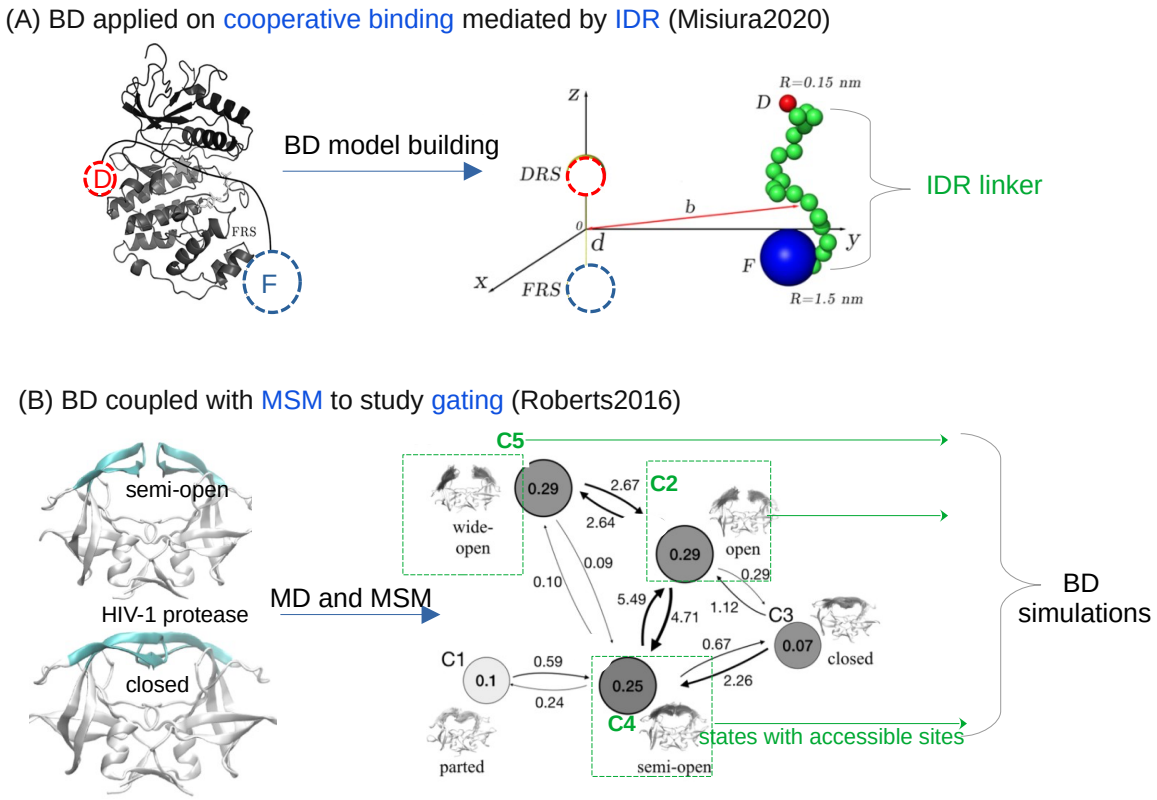

(A) BD applied on cooperative binding mediated by IDR (Misiura2020)
D
FRS
F
BD model building
z
DRS
R=0.15 nm
D
IDR linker
b
d
y
x
FRS
F
R=1.5 nm
(B) BD coupled with MSM to study gating (Roberts2016)
semi-open
HIV-1 protease
closed
MD and MSM
C5
0.29
2.67
C2
2.64
wide-open
0.29
open
0.09
0.29
0.10
5.49
4.71
1.12
C3
0.67
0.07
closed
C1
0.59
0.1
0.25
2.26
0.24
C4
parted
semi-open
states with accessible sites
BD simulations
(C) BD for scaffolded sequential binding events (Sadiq2021)
H2O2
GOx
HRP
q
b
H2O2
(D) BD for crowded binding events (Wieczorek2008)

Figure 6: .

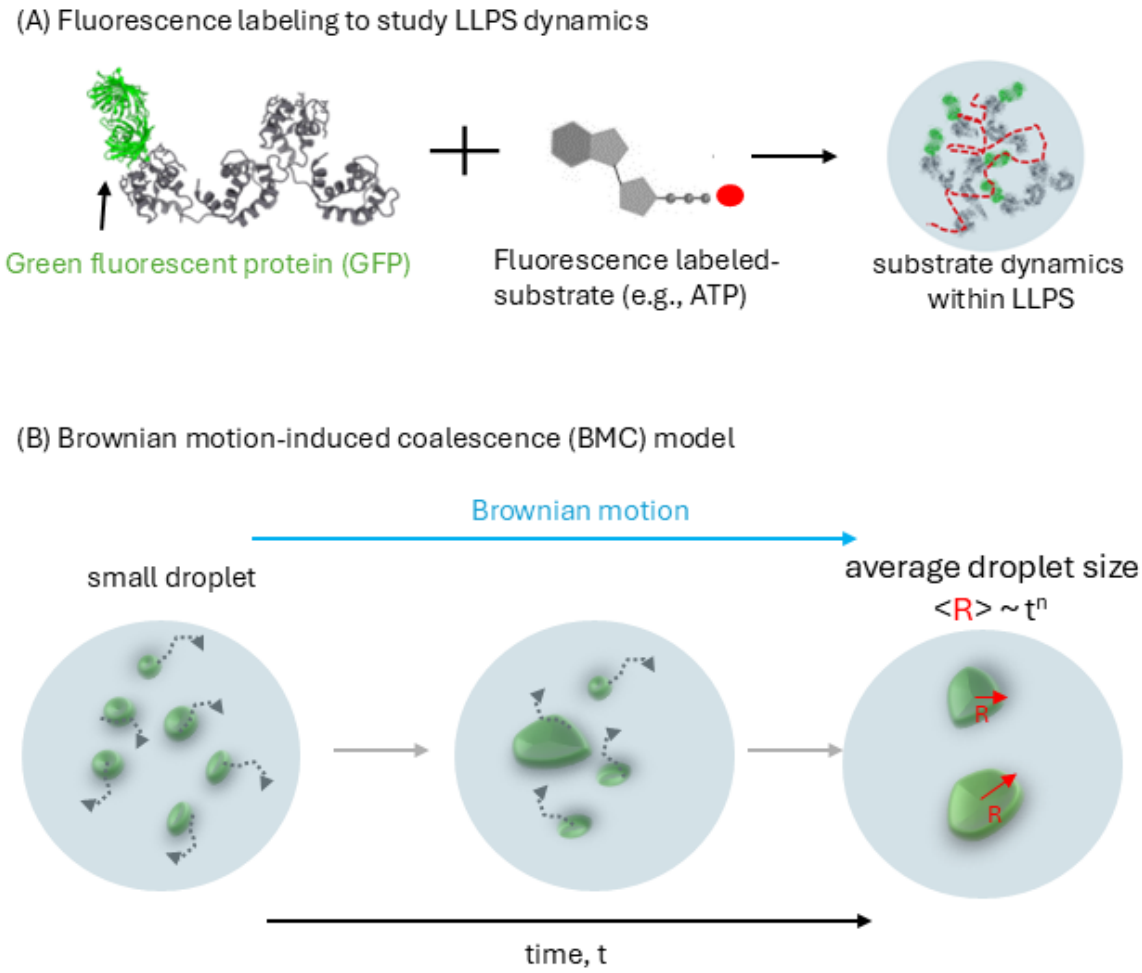

(A) Fluorescence labeling to study LLPS dynamics
Green fluorescent protein (GFP)
Fluorescence labeled-substrate (e.g., ATP)
substrate dynamics within LLPS
(B) Brownian motion-induced coalescence (BMC) model
Brownian motion
small droplet
average droplet size
<R> ~ tⁿ
R
R
time, t

Figure 7: .

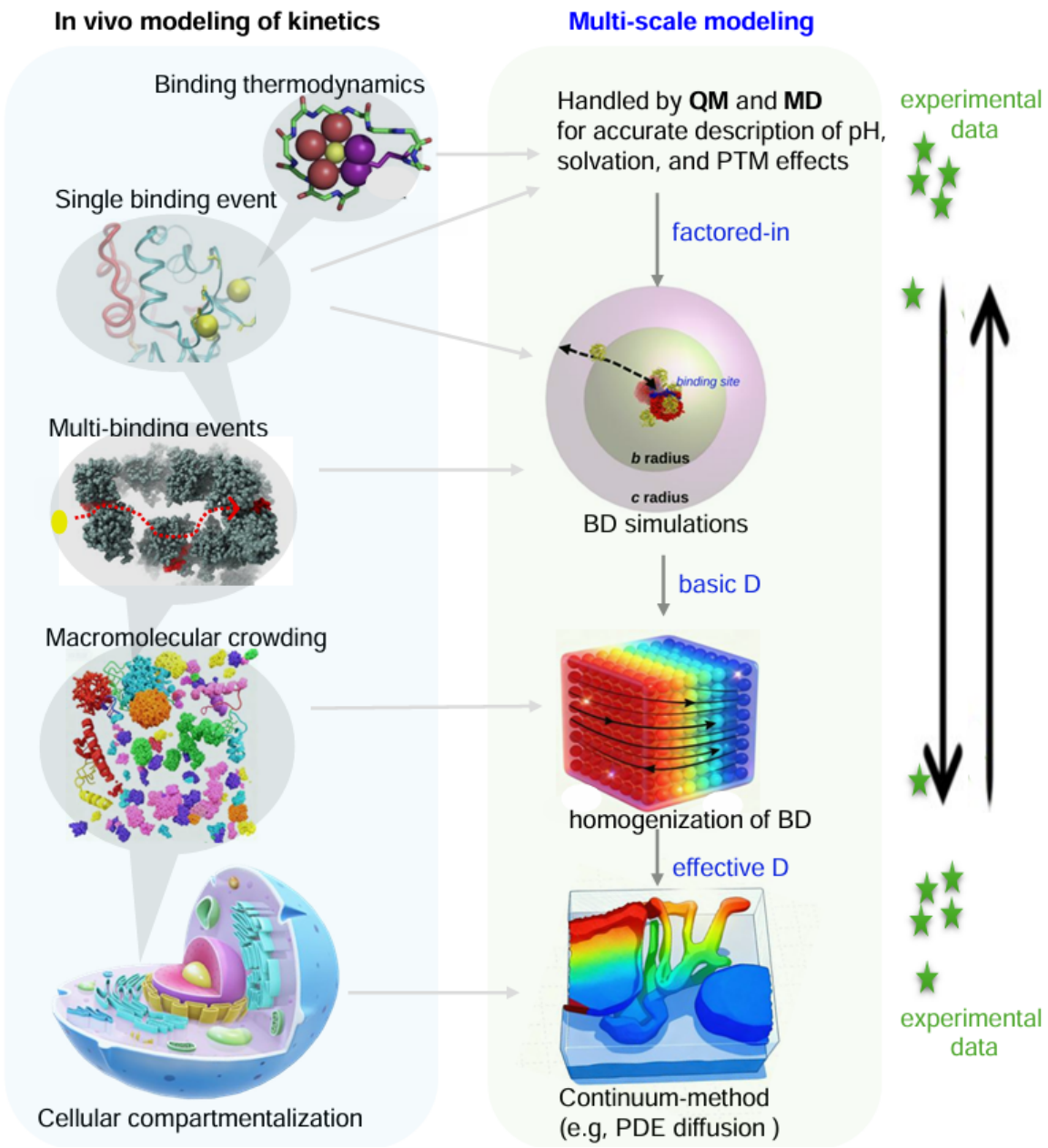
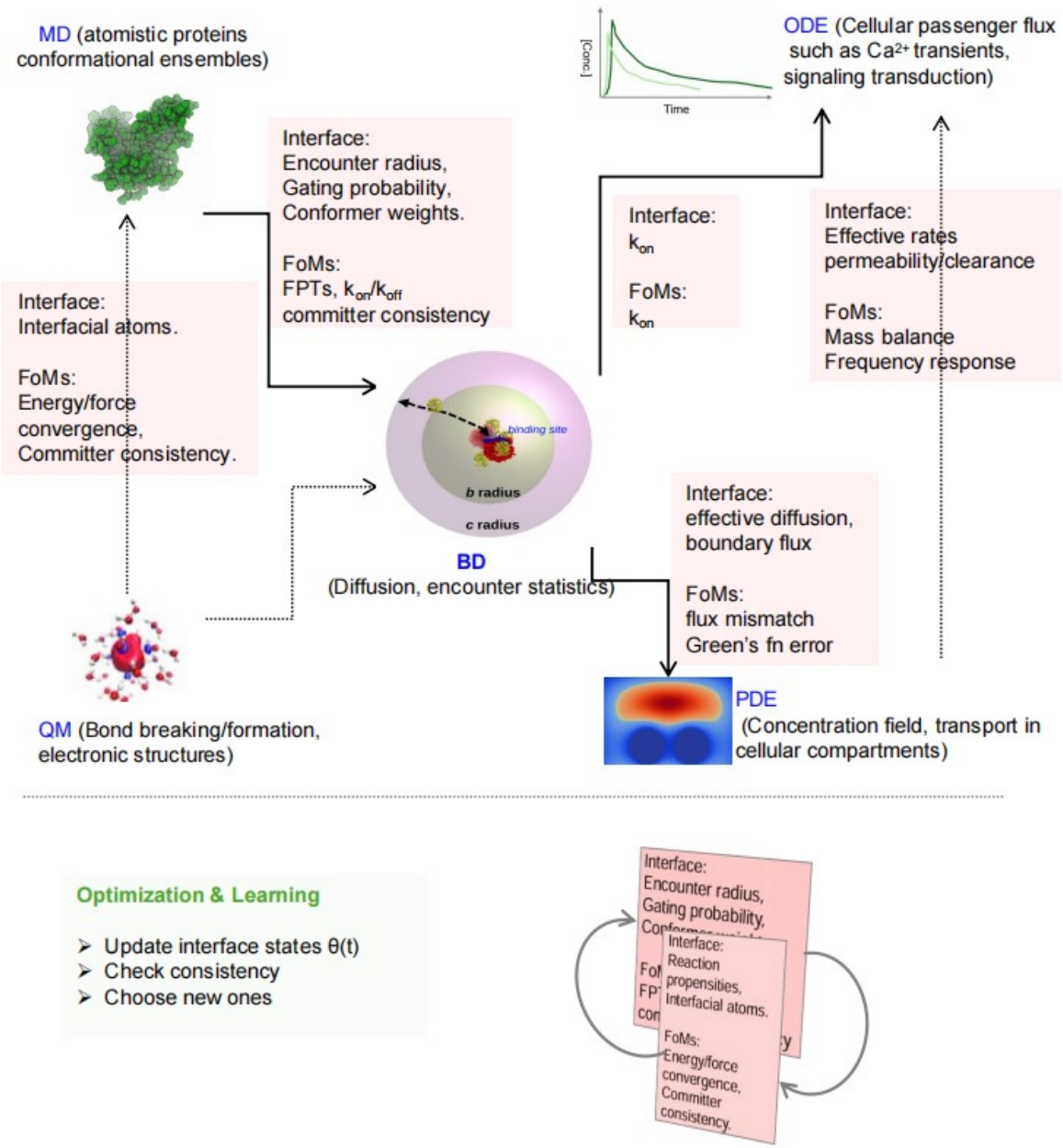

In vivo modeling of kinetics
Multi-scale modeling
Binding thermodynamics
Handled by QM and MD
for accurate description of pH,
solvation, and PTM effects
experimental
data
Single binding event
factored-in
binding site
b radius
c radius
Multi-binding events
BD simulations
basic D
Macromolecular crowding
homogenization of BD
effective D
experimental
data
Cellular compartmentalization
Continuum-method
(e.g, PDE diffusion)

Figure 8: .


MD (atomistic proteins conformational ensembles)
Interface:
Encounter radius,
Gating probability,
Conformer weights.
FoMs:
FPTs, k_{on}/k_{off}
committer consistency
Interface:
Interfacial atoms.
FoMs:
Energy/force convergence,
Committer consistency.
binding site
b radius
c radius
BD
(Diffusion, encounter statistics)
QM (Bond breaking/formation, electronic structures)
[conc.]
Time
ODE (Cellular passenger flux such as Ca^{2+} transients, signaling transduction)
Interface:
k_{on}
FoMs:
k_{on}
Interface:
Effective rates permeability/clearance
FoMs:
Mass balance
Frequency response
Interface:
effective diffusion,
boundary flux
FoMs:
flux mismatch
Green's fn error
PDE
(Concentration field, transport in cellular compartments)

Optimization & Learning
Update interface states θ(t)
Check consistency
Choose new ones
Interface:
Encounter radius,
Gating probability,
Interface:
Reaction propensities,
Interfacial atoms.
FoMs:
Energy/force convergence,
Committer consistency.

Figure 9: .

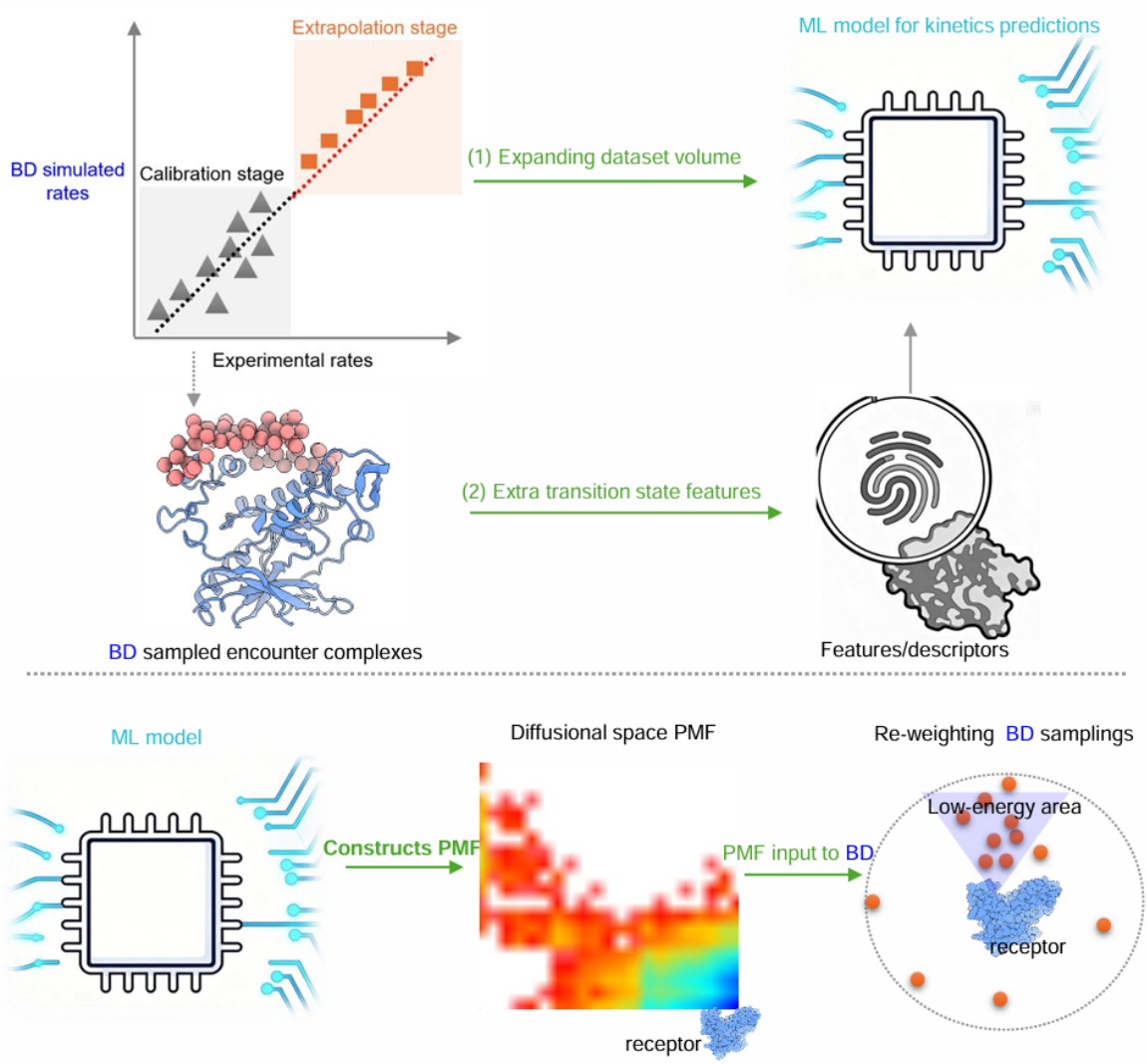

Extrapolation stage
ML model for kinetics predictions
BD simulated rates
Calibration stage
(1) Expanding dataset volume
Experimental rates
(2) Extra transition state features
BD sampled encounter complexes
Features/descriptors
ML model
Diffusional space PMF
Re-weighting BD samplings
Constructs PMF
PMF input to BD
Low-energy area
receptor
receptor

Figure 10: .

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