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PERSPECTIVE

Molecular motions in drug design: the coming age of the metadynamics method

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Abstract Metadynamics is emerging as a useful free energy method in physics, chemistry and biology. Recently, it has been applied also to investigate ligand binding to biomolecules of pharmacological interest. Here, after introducing the basic idea of the method, we review applications to challenging targets for pharmaceutical intervention. We show that this methodology, especially when combined with a variety of other computational approaches such as molecular docking and/or molecular dynamics simulation, may be useful to predict structure and energetics of ligand/target complexes even when the targets lack a deep binding cavity, such as DNA and proteins undergoing fibrillation in neurodegenerative diseases. Furthermore, the method allows investigating the routes of molecular recognition and the associated binding energy profiles, providing a molecular interpretation to experimental data.

Keywords Docking · DNA minor groove · Prion Protein · Metadynamics

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Introduction

Communication in the cellular framework is fundamental for life organization and survival. It involves a complex network of signaling pathways among different macromolecules, such as metabolites, proteins and nucleic acids. Recent advances in genomics and proteomics techniques have allowed building up wide networks of protein–protein and protein–DNA interactions for different pathways [1, 2]. Alterations in such regulation networks are usually responsible for the outcome of many diseases. Moreover, interfering with a key target in a given pathway may result in effective re-adjustment of a disease-altered cellular route. Indeed, drug action is most often based on the interference with one or more cellular pathways [3, 4].

Novel powerful drug design strategies are likely to be founded on a comprehensive knowledge of these interacting networks at different levels, ranging from a rationalization of the connections between network edges and their regulatory paths (Systems Biology) to an understanding of the biophysical determinants of association between macromolecules (Molecular Recognition). In particular, understanding the physical chemistry underlying biomolecular interactions is crucial to design rationally compounds interfering with cellular targets [5–7]. In response to this need, new experimental [8–10] and computational [6, 11] methodologies are being continuously developed.

In the field of computational science, molecular modeling of drug-target interactions based on the 3D structure of cellular components (e.g., proteins or DNA/RNA) is of enormous help in drug design. Current protocols exploit the exquisite shape and chemical complementarity between a compound (e.g., small organic molecule) and the corresponding macromolecular target. Computational tools, such as virtual docking, allow predicting, in a very short

time, the pose of a ligand binding to its macromolecular target based only on the three dimensional atomic structures of both [12–14]. Software packages based on this approach (such as FlexX [15], Surflex [16], Glide [17, 18], GOLD [19], DOCK [20], AUTODOCK [21, 22], ATTRACT[23]) are successfully used in early stages of drug discovery to pre-screen large datasets of compounds, reducing largely the number of compounds to be synthesized and tested experimentally in the subsequent stages. They are also very useful in investigating the effect of mutations on the binding event [24].

There are, however, aspects that are neglected or largely approximated by standard docking protocols, limiting their reliability in predicting binding poses. These limitations are mainly due to the inappropriate treatment of molecular flexibility and solvent mediated binding [6, 25–30], both entering in a crucial way in the thermodynamics and the kinetics of molecular recognition [7, 31]. Nowadays, most packages account for ligand flexibility. Target flexibility, though, is still a major challenge [21, 32–41]. Fluctuations of the target molecule can be taken into account prior to, during, and after the docking procedure. Prior to docking, multiple protein conformations can be selected from different crystal and NMR structures or calculated using computational sampling methods such as molecular dynamics, normal modes analysis, essential dynamics, and loop modeling [6, 42–47]. During the docking procedure, the flexibility is well thought-out by using soft potentials in the scoring functions, or by the exploration of rotamer libraries to simulate side-chain movements [32, 41, 48]. New promising approaches of flexible refinement, ensemble docking and explicit inclusion of flexibility have been developed, in particular in the field of protein–protein docking [44, 49, 50].

Anyhow, these representations of target flexibility and fluctuations are decoupled from the binding process itself. This may alter the final structure of the complex and the predicted binding affinity, limiting the predictive power of these methods [26, 28, 51–54].

After docking procedure, Molecular Dynamics (MD) based techniques are an alternative approach. They describe the dynamics of intermolecular interactions based on well-founded physics fundamentals. They implicitly take into account the conformational flexibility of the receptor in (typically) tens or hundreds nanosecond timescales. Additionally, solvation effects on the structure/dynamics of the interacting macromolecules and on the binding event itself can be described explicitly. Statistical mechanics methods based on MD can provide accurate prediction of binding free energies (binding affinities), yet to the price of much larger computational cost. Even in that case, however, one has to keep in mind that conformational changes are slow movements that may accompany ligand binding to its target receptor [55]. These transitions, if not described properly, can affect the accurate prediction of the final drug-target complex structure and their binding strength.

In this Review, we focus on a recently introduced statistical mechanics approach, the so-called metadynamics [56]. This allows extending the simulation time-scales for slow processes and in particular investigating molecular recognition between ligands and their targets [57–65]. The basics of this methodology are firstly introduced after recalling to standard docking concepts and free energy simulation techniques. Two applications of metadynamics to biomolecular recognition are then reviewed. Finally, the current limitations and expected further developments of this methodology are discussed in the outlook section.

The method

From docking to free energy calculations

Standard molecular docking protocols (MDPs) allow predicting the three dimensional structure of macromolecular complexes as well as their binding affinity at a low computational cost. These protocols require information about the structure of the receptor and the ligand as well as the presumable interfacing region between them [66]. They are based on a step-wise process. First, a proper *search algorithm* generates various configurations of the ligand within the binding site of the target. In the second step, each configuration is evaluated and ranked according to an *energy function*. These energy functions are usually based on effective potentials that are heuristically trained to reproduce experimental binding affinities of validated training data sets of ligand-target complexes. This simplicity makes standard MDPs computationally cheap. It allows them to be used as a tool for virtual drug screening. In fact, these methods are routinely used in early stages of drug discovery to pre-screen large datasets of compounds. Thus, a remarkable decrease in the number of compounds to be synthesized and tested experimentally in the subsequent stages is achieved. However, molecular plasticity and solvation effects (Fig. 1) are not or only approximately taken into account in these approaches [28, 54].

Free energy simulations may be then used to investigate the molecular association process and to predict binding affinity (for a statistical-thermodynamic basis for them, see, e.g., Ref. [67]). Different classes of computational methods can be used [68, 69]. These include among the others: Thermodynamic Integration [70], MM/PBSA and MM/GBSA [71], Adaptive Bias Force Steering [72, 73], Linear Interaction Energy [74], Steered Dynamics[75, 76], Metadynamics [56, 77], Umbrella Sampling [78]. Such

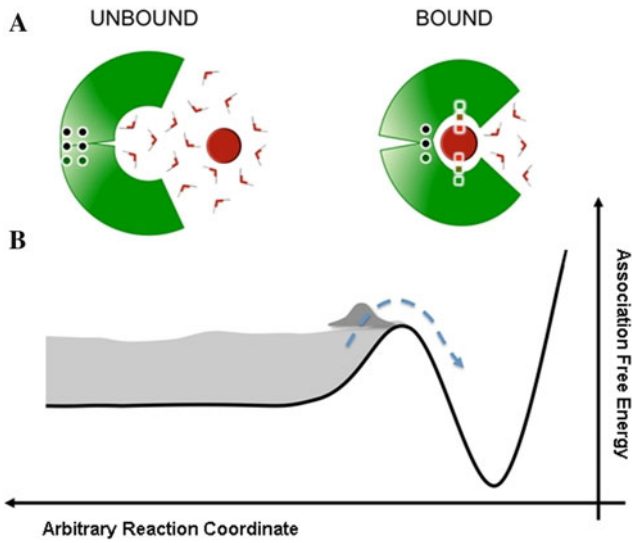

Fig. 1 **a** Schematic representation of the molecular association process: a recognition event involving flexible partners in aqueous solution. When a ligand (*red sphere*) binds to a flexible macromolecule (*green*), this latter undergoes a conformational change in order to optimize inter- and intra-molecular contacts (*colored* and *black dotted lines* respectively). The network of waters surrounding macromolecules (*red-white sticks*) also rearranges during the process. **b** One-dimension free energy profile of the molecular association process as a function of an arbitrary reaction coordinate that takes into account all the essential degrees of freedom. Upon sequential addition of hills (*gray-colored filling*) during a metadynamics simulation the unbound basin is filled until the system has enough energy to overpass the free energy barrier to the bound basin

methods treat explicitly the conformational flexibility of the receptor and also may take into account water solvation of the system. Details on these approaches are out of the scope of this review and might be found in the references reported above. In the following, more details are given on metadynamics, the approach extensively used in the case studies reported here.

Metadynamics

This is a MD-based technique aimed at enhancing the sampling of the configuration phase space during a simulation and at estimating free energy landscapes [56]. The method is based on a dimensional reduction: it requires the previous definition of a set of collective variables (CVs) describing all the relevant transitions of the process under study. During an MD simulation small "hills" (Gaussian-like repulsive potentials) are added with a defined frequency on the explored regions of the CV space, and the resulting "meta-force" is added to the forces acting on the atoms of the system during a standard MD simulation. These repulsive potentials in the end disfavor the system to re-visit already sampled points (Fig. 1). The method can be exploited for accelerating "rare" events such as molecular recognition (where "rare" refers to the long timescale of the association/dissociation events, compared with the times reached by standard all-atoms MD simulations), and has the advantage of mapping the free energy surface of the simulated process. Indeed, it has been demonstrated that the negative of the sum of the repulsive Gaussian potential terms approximates the underlying free energy as a function of the chosen CVs [77]. The system can evolve towards unpredicted stable or metastable states not presumed previously. Indeed, due to the "basin-filling" character of metadynamics, the system naturally evolves along the more convenient path (in terms of free energy barriers) if used appropriately [79].

Two key issues must be pointed out. First, the choice of a proper set of CVs is essential; typical CVs successfully used to study molecular recognition include general variables, such as coordination numbers, hydrogen bonds, non-polar contacts, distances, angles, dihedral angles, RMSD [80, 81], and more sophisticated ones, such as cation-π or electrostatic interaction energies [81]. Second, the shape of the Gaussian-like potentials added onto the CVs space. As a rule of thumb, the height of the Gaussian term should be $\sim 1/10$ of the expected activation energy of the process, and the width should be $\sim 1/3$ of the fluctuations of the system along the considered CV in standard MD. Gaussian terms of which the height exceeds the threshold above indicated might induce structural rearrangements that are artifacts due to the excess of energy flowing in the system. On the other side, too low Gaussian terms might slow down dramatically the simulation making the rare event not observable during the simulation. When simulating typical biological systems containing 10,000–1,00,000 atoms, it is convenient to introduce new Gaussian terms to the bias potential every 10–50 ps. In this way the perturbation introduced upon addition of a new term is small and will be readily absorbed by the system, with the latter evolving in a quasi-equilibrium condition. A way to estimate the error on the free energy profile reconstructed from metadynamics simulations has been proposed [81]. Typical errors are in the range of 1–2 kcal/mol. Recently a new theoretical frame has been provided which allows for a rigorous control of convergence and errors [79].

One way to ensure a correct description of the process in a metadynamics simulation is to enlarge the number of CVs. Unfortunately this number cannot be arbitrarily large as the computational cost to reconstruct higher dimensional free energy surfaces grows exponentially with the number of CVs [81]. For this reason metadynamics has been recently combined with different techniques in order to allow for more efficient reconstruction of multidimensional free energies (multiple walkers [82], parallel tempering, [83], path sampling [84], bias-exchange [80]). A discussion of them, in light of biological application, is offered in Ref.

[64]. Here we mention one of these extensions, bias-exchange metadynamics, for which we describe an application. In this approach, multiple replicas of the system run in parallel under the effect of different low-dimensional metadynamics biases (dependent on different sets of CVs). In this way, different replicas explore different regions of the space. Exchanges of the biases are performed at different times of the simulation according to a Metropolis acceptance criterion. This enhances the exploration of the phase space in the multiple CVs chosen. A weighted histogram based procedure has been proposed to reconstruct the multidimensional free energy landscapes from the lower-dimensional metadynamics biases. This methodology has allowed studying complex processes such as peptide folding [83, 85–88] and macromolecular association [58, 89].

Applications

Metadynamics has been successfully applied to study a large number of processes in the fields of physics, chemistry, and biology [62, 63, 84, 90]. Applications of metadynamics to the study of biomolecular processes have been recently reviewed [64]. Several groups have applied this technique to study drug-receptor binding in different systems [57, 59–62, 89, 91]. Most of these applications dealt with ligand binding to specific cavities of the target. Here we focus on the somehow difficult situation in which multiple binding sites exist within the same pocket, or deep binding sites are lacking. For the first case, we report here the study of the molecular recognition between DNA and two minor groove binders [60]. In particular, we used metadynamics to describe relevant intermediates of the dissociation process and to get reliable estimates of the associated kinetics.

For the second case study, simulations of ligands binding to protein surfaces have been hampered by a lack of structural information of the protein–ligand complex. We also report here an enhanced molecular docking (EMD) approach, based on metadynamics, that was used to investigate the association between PrP^C and one of its cognate ligands [89].

DNA molecular recognition

DNA represents a target for several therapies. In particular organic molecules targeting the minor groove with different sequence selectivity are promising multifunctional compounds for genetic-tuned therapy [92–94]. Molecular recognition of DNA by these drugs is a complex process that may involve several steps before formation of the most stable adduct: both non-covalent and covalent binders may exploit mechanisms like one-dimensional sliding within the minor groove and translocation among different sites (involving binding and dissociation) [95–98]. Thus, molecular recognition of DNA is a typical complex process where flexibility of the target and solvation effects play a major role in recognition. Understanding the kinetics of the processes offers key information for supporting an efficient drug design. In particular, the importance of dissociation for the selectivity and the affinity of different minor groove binders has been pointed out by several experimental studies [99–102]. Metadynamics represents a reliable tool to investigate such processes. We used it to build a microscopic picture of the dissociation from DNA minor groove of two molecules, the distamycin A and the anhydrous form of anthramycin (hereafter DST and IMI,

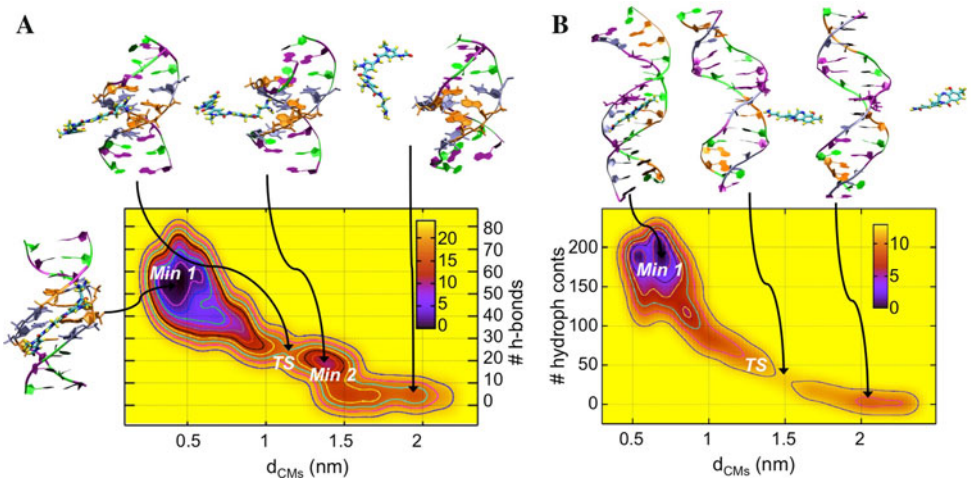

Fig. 2 **a** Free-energy surface $G(d_{CMs}\ n_{hph})$ associated with the detachment of DST from DNA. Isosurfaces are drawn one per 2 kcal/mol. Relevant conformations are also reported, corresponding to the main minimum, barrier, and dissociated ligand. **b** Free-energy surface $G(d_{CMs}\ n_{hb})$ associated with the detachment of IMI from DNA. Adapted from Ref. [60]

respectively) [60]. While DST, the prototype of noncovalent binders, is positively charged, quite flexible and highly selective towards AT-rich tracts, IMI is an alkylating agent, neutral, rather rigid and exhibits only a modest preference towards PuGPu triplets. The Free Energy Surface (FESs) related to the unbinding process of DST and IMI are reported in Fig. 2a, b, respectively. Metadynamics allowed characterizing how differences in structural and chemical features reflect on the mechanism of dissociation and on the associated free energy profile. We found that the dissociation path of DST has a stable intermediate (Min2 in Fig. 2a), in which the positively charged amidinium tail of DST complements the negative electrostatic potential of the $d[ATTA]_2$ tract. The effective free-energy barrier associated with the removal of DST from DNA extracted from our FES ($\sim$16.5–18 kcal/mol) agree well with the value of 16.6 kcal/mol derived from stopped-flow experiments [101]. In addition, these results are comparable with kinetic data for the dye Hoechst 33258, a prototype of minor groove non-covalent binders containing benzimidazole. Indeed, stopped-flow experiments on the molecular recognition of DNA segments by this ligand have been interpreted on the basis of a model that requires the presence of a bound intermediate, structurally different from the fully bound complex, and from which the rates of binding and dissociation are very similar [103]. This situation is reminiscent of our findings for DST: an intermediate featuring the ligand partially bound to the DNA duplex exists, for which the free energy barriers for association or dissociation are very similar. In contrast to DST, IMI encounters a single transition state during its escaping from the minor drug, as shown in Fig. 2b. The lack of stable intermediates might be traced back to the fact that the tail of the molecule, although polar, is neutral. Additionally, the presence of IMI produces a larger local widening of the minor groove than for DST, allowing for an extended hydration of the whole region between IMI and one strand.

This example highlights how metadynamics can be used to find common mechanisms and striking differences in non-covalent recognition by diverse minor groove binders. In addition, if properly used the method is able to provide reliable insights into the kinetics of the dissociation process.

Ligand–prion protein interactions

A promising improvement in the study of biomolecular association on protein surfaces has been recently addressed in our group in the framework of small organic molecule interacting with a cavity-less protein [89]. We proposed a novel protocol, called EMD (enhanced molecular docking) which combines standard docking calculations with free energy simulations based on metadynamics (Fig. 3). This provides a step forward towards the development of a tool able to identify small organic molecules that interfere with cavity-less proteins.

We applied this protocol to the study of ligand **GN8** [104] (2-pyrrolidin-1-yl-N-[4-[4-(2-pyrrolidin-1-yl-acetylamino)-benzyl]-phenyl]-acetamide) binding to the PrP^C, the main agent involved in prion diseases [105]. Recently, the ligand **GN8** has been shown to bind PrP^C in the micromolar range ($K_d = 3.9$ μM, corresponding to a standard free energy of binding $\Delta G_0 = -7.5$ kcal/mol) [104]. Given the lack of deep binding pockets along the protein structure of PrP^C, it is reasonable to assume that **GN8** will not bind specifically to a single site. This hypothesis was also encouraged from NMR data, which

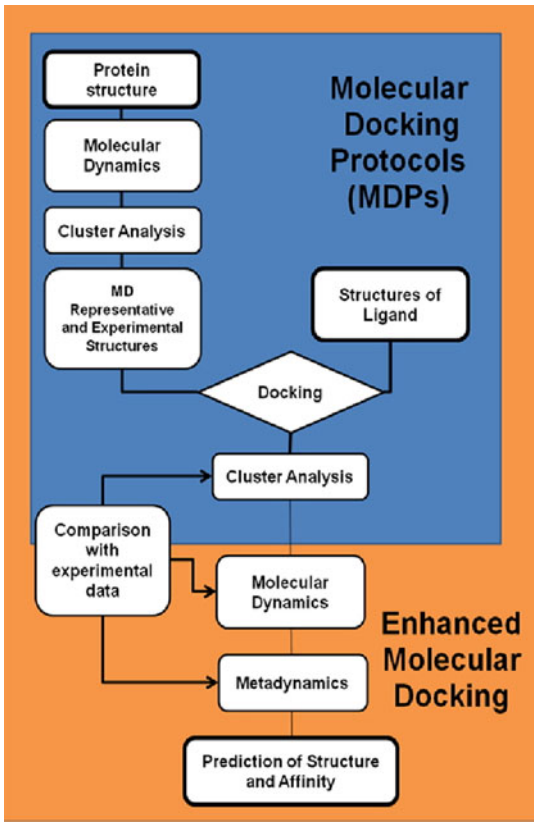

Fig. 3 Molecular Docking Protocols (MDPs) are used to guess putative ligand binding regions on target surfaces, based on structural information on the two separated moieties. Structural information on the target may come from NMR or X-ray studies and, in some cases, also from molecular simulation. Ligand may be docked onto the entire structure or on a putative binding site. Cluster analysis is used to group MD conformers and/or ligand/target adducts into representative structures. In the Enhanced Molecular Docking (EMD) approach, MD simulations may be used to relax the structures and to investigate the role of hydration. Enhanced sampling simulation techniques in explicit solvent (here metadynamics) allow to explore the ligand binding space and to predict free energy of binding

enables several residues located on distant regions of PrP^C surface interacting with **GN8** to be identified [104].

Our proposed EMD protocol was applied to identify the PrP^C sites where **GN8**, in its different protonation states (0, +1, +2), preferentially binds. The protocol combines standard MDP with metadynamics. As in any MDP, MD simulations were used initially to relax the protein structure in aqueous solution. Secondly, representative PrP^C structures were selected by statistical analysis of the MD simulation. Standard docking calculations were then used to dock the ligand **GN8** on these protein structures, providing a first guess of putative binding regions. MDP was not sufficient to explain all distant contacts reported by NMR [104]. Successively, metadynamics was used to simulate the **GN8** binding process from solution to PrP^C surface. This requires the definition of a set of CVs (see Sect. *Method*). In our proposed EMD, CVs are selected from analysis of the standard MDP outcomes. These simulations allowed us to identify alternative binding poses of **GN8** onto PrP^C surface and also to predict the binding affinity. These predictions were comparable to experimental data obtained via NMR chemical shift perturbations and affinity measurements. We observed a multiple binding site pattern of **GN8** complementing that proposed by Kuwata et al. [104]. Taken altogether, these provide a structural basis for the experimental NMR-contacts (see Fig. 4). These contacts are observed in distant positions of PrP^C: in fact, **GN8** may bind in opposite parts of the protein surface. This result was not predictable a priori and could only be obtained when MDPs are extended with enhanced sampling simulations. This example represents a successful application of a promising tool able to identify small organic molecules that interfere with cavity-less proteins, in which water-mediated interactions become important, and the ligand can bind at the same time in multiple sites of the protein surface.

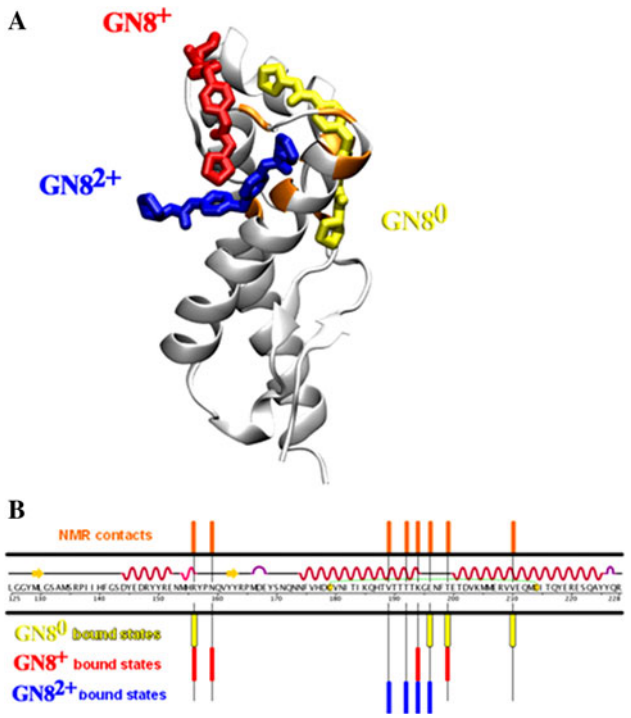

Fig. 4 **a** Superimposition of 1^0 (*yellow*), 1^+ (*red*) and 1^{2+} (*blue*) binding poses. **b** Human PrP^C sequence. The residues experimentally found to be involved in binding are highlighted in *orange bars* [104]. Those emerging from the calculations are shown with the same color code as (**a**). Adapted from Ref. [89]

Outlook

Computer-aided ligand design, based on the structure of both the ligand and the target, is a cornerstone of drug discovery. Thanks to the simplicity in use and the computational effectiveness, standard molecular docking protocols are routinely used in this field to predict the formation of ligand-target complexes. The cheap computational cost of these methods comes at expenses of a less accurate description of the thermodynamics and kinetics of the association process. In this context, molecular simulation based protocols have shown their ability to catch and to describe at a good level of accuracy the pharmacologically and biophysically relevant features of the ligand-binding process, even in challenging cases such as interactions with nucleic acids and protein surfaces. However, this more accurate description of the molecular association process requires much more computational resources than standard molecular docking protocols do. For this reason, such methodology is not currently properly suited for routinely virtual screening of compounds on a given target. In spite of this, the continuous progress in algorithms, combined with the power of parallel computing, allows us to suggest that in a not too far future such approaches can be exploited as an important guide tool for the design of new therapeutic agents.

In addition to the computational cost limitation, other reasons that still prevent the use of metadynamics as a drug design technique may be the existence of multiple possibilities of running a metadynamics simulation, and the absence of a unified and automatized procedure to prepare, execute and analyze the simulation. The EMD protocol introduced in Ref. [89] and that we have reviewed here together with other applications was, indeed, an attempt to set up a standard procedure to carry out such kind calculations in the field of target based ligand design.

A big effort has been made to unify all the current capabilities of the metadynamics methodology in a unique software package (named PLUMED, http://merlino.mi.infn.it/~plumed/PLUMED/Home.html) that easily integrates into most widely used molecular dynamics simulation

software such as GROMACS, NAMD, AMBER, LAMMPS and DL-POLY. This certainly simplifies the execution of metadynamics and opens the access to more scientists interested in this approach. The set-up of the simulation, however, still remains a user-dependent step. There is not an exact rule for setting up the metadynamics parameters (number and type of collective variables, size of the hills, time deposition, final simulation time, etc.). We have provided some hints on how to set-up these parameters in the *Method* section. Regarding the analysis of the resulting simulations, a graphical-user-interface is under development that will simplify the tasks to be performed in order to extract structural, thermodynamic and kinetic data from a molecular dynamics simulation, and in particular metadynamics (A. Laio, personal communication).

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