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Article *in* Current Topics in Medicinal Chemistry · January 2019

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REVIEW ARTICLE

Hotspot Identification on Protein Surfaces Using Probe-Based MD Simulations: Successes and Challenges

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ARTICLE HISTORY

Received: August 17, 2018
Revised: September 21, 2018
Accepted: November 21, 2018

DOI:
10.2174/1568026619666181130120308

Abstract: Molecular Dynamics (MD) based computational co-solvent mapping methods involve the generation of an ensemble of MD-sampled target protein conformations and using selected small molecule fragments to identify and characterize binding sites on the surface of a target protein. This approach incorporates atomic-level solvation effects and protein mobility. It has shown great promise in the identification of conventional competitive and allosteric binding sites. It is also currently emerging as a useful tool in the early stages of drug discovery. This review summarizes efforts as well as discusses some methodological advances and challenges in binding site identification process through these co-solvent mapping methods.

Keywords: Binding site identification, Co-solvents, fragment-based, Probe-based, Hotspots, Structure-based drug design.

1. INTRODUCTION

Identification of ligand binding sites on the surface of a target protein is a key step in Structure-based Drug Discovery (SBDD) [1-4]. This can be realized by multiple experimental and computational techniques. Examples of experimental SBDD techniques are ligand-based NMR spectroscopy [5], fragment-based NMR spectroscopy [6-8] and multi-solvent crystallography [9-11]. These techniques are robust and readily sample the dynamical nature of protein structures. However, they are time-consuming and expensive when a large number of ligands/fragments are considered for screening. Conversely, computational SBDD techniques offer a cost effective mean that complement the aforementioned experimental techniques by efficiently predicting putative ligand binding sites based on a large number of fragments or ligands. These computational predictions guide subsequent efforts and allow to experimentally focus on the most relevant binding sites, fragments or ligands. Examples of computational SBDD methods include blind docking [12-15], SEED [16], FTMAP [17, 18], LIBSA [19] and SiteMap [20]. These methods are usually utilized to detect binding sites throughout the entire surface of the target protein. However, they rely on static-average of experimental structures for which important binding sites might be buried. In the absence of experimental target structures, ligand-based drug design methods such as structure activity relationship analysis [21-24] or pharmacophore modeling [25-27] are alternatively used.

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While, molecular dynamics (MD)-based computational methods are used to overcome the limitation of using a static-structure in addressing flexibility of binding sites [28] and accounting for other interactions that are stabilized by explicit water molecules. Examples of these MD methods include relaxed complex scheme(RCS) [29-31], probe-based molecular dynamics (pMD) [32-37], accelerated [38] MD simulations, high throughput MD simulations [39, 40], Wrap 'n' Shake (WnS) [41] and MDpocket [42]. In RCS, MD simulations are used to generate an ensemble of target conformations that are afterward used in blind dockings to identify binding sites on the surface of each conformer [43]. Wrap 'n' Shake method use a modified-blind docking step to wrap a monolayer of ligand around the target surface, followed by a short MD simulation to shake off ligands that are loosely bound. MDpocket also uses MD generated ensemble of conformers to identify binding sites and surface grooves that might be transiently formed in the protein based on geometrical analysis. pMD simulation methods are the subject of this review, a detailed description of these methods and their application is presented in subsequent paragraphs and sections. We will also briefly discuss both accelerated and high throughput MD simulations in the conclusions section.

Earlier MD simulations of proteins in the presence of co-solvent molecules were focused on the thermodynamics and global interaction of the co-solvents with the protein [44-47]. In this review, we focus on more recent MD simulations of the target protein in the presence of small organic molecules to identify and characterize ligand binding sites. In the latter studies, the small molecules are usually chosen from fragmentation of drug leads into smaller pieces containing discrete functional groups. These small molecules are then used as probes in MD simulations along with a targeted protein to

1873-4294/18 $58.00+.00

delineate ligand binding sites on its surface. The identification of a binding site and its druggability are then linked to the probability of contact between probe and protein atoms. Furthermore, different probe-surface contact profiles can be subsequently incorporated to assemble ligands with higher binding affinities. These computational solvent mapping methods incorporate atomic-level solvation effects and protein mobility and have been described in the literature under various names including probe-based MD (pMD) [32, 48, 49], mixed-solvent MD (MixMD) [50-53], solvent competition [54], co-solvent MD [55-57] and ligand competitive saturation [35, 58-60]. A major advantage of these methods is that they rely on physics-based simulations and not on a knowledge-based scoring function. Throughout this review, we will refer to all atom explicit solvent MD simulations of a protein in the presence of small organic molecules (also called alternatively co-solvents, fragments or probes) as pMD methods.

2. IDENTIFICATION OF LIGAND BINDING SITES FOR SOLUBLE PROTEINS

A large number of pMD studies have been already reported in the literature [32-37, 48-55, 57-64]. These studies have mostly focused on reproducing known active and allosteric binding sites of many pharmaceutically important soluble protein targets. In the following, we summarize some of the key studies.

Seco *et al* [34] reported for the first time a method that incorporates co-solvents in an all-atom explicit solvent MD simulation to identify ligand binding sites and their druggability. The advantage of this method is that it depends on first-principles simulations and not trained on a data set. They have demonstrated that pMD simulation in the presence of isopropanol capture the allosteric/active binding sites of important pharmaceutical targets such as MDM2, LFA1, Protein phosphatase 1B (PTP-1B), P38 and androgen receptor (AR). Guvench and MacKerell [35] similarly reported a similar method that took the advantage of using small organic molecules in an all-atom explicit solvent MD simulation to map out binding sites on a surface of a target protein. They termed it SILCS (Site Identification by Ligand Competitive Saturation) method which was demonstrated on the BCL-6 protein by reproducing its biologically relevant conformational changes and interactions in the binding pocket of the non-homologous SMRT and BCOR peptides. Yang and Wang [57] showed that MD simulations of either the apo- or holo-crystal structures of the Bcl-xL in pure water did not yield conformations found in the crystal structures of Bcl-xL in complex with its binding partners due to hydrophobic collapse of the binding site. On the other hand, pMD simulations of either the apo- or holo-crystal structure of the Bcl-xL in the presence of isopropanol as a co-solvent yielded binding-site conformations similar to that found in the co-crystal structures of Bcl-xL. Other hydrophobic binding hot spots identified using the conformations from the co-solvent simulations of the inactive apo-Bcl-xL conformation were consistent with experimental structural data of known inhibitors. Ung and coworkers [50] used pMD simulations to study human immunodeficiency virus type-1 protease (HIVp) in the presence of acetonitrile, isopropanol and pyrimidine at low and high concentrations. Remarkably, significant probe occupancies were observed in the catalytic site and potential allosteric sites leading to a more robust identification of experimentally confirmed ligand binding sites, especially in the lower concentration simulations. In particular, a putative allosteric binding site underneath the flap of HIVp, has been confirmed by the presence of a 5-nitroindole fragment in a crystal structure. Bakan and colleagues [36] carried out several extensive pMD simulations of murine double mutant-2, protein tyrosine phosphatase 1B (PTP1B), lymphocyte function-associated antigen 1, vertebrate kinesin-5 (Eg5), and p38 mitogen-activated protein kinase, in the presence of a acetamide, isopropylamine and acetic acid. The choice of these small organic molecules was based on water solubility and their frequency of occurrence as substructures in FDA-approved and experimental drug molecules. To reduce the noise due to observed spurious probe hotspots, they combined proximal interaction spots of different probe to predict maximal achievable ligand binding affinities. Their analysis of pMD-derived probe interaction spots of known drugs for a variety of targets, captured known active/allosteric ligand binding sites and affinities. Their analysis of pMD-derived hotspots suggested additional novel druggable binding sites along with their induced structural changes upon ligand binding. Ghanakota and Carlson [51] demonstrated in a study of different target proteins (ABL kinase, Androgen receptor, CHK1 kinase, Glucokinase, PDK1 kinase, Farnesyl Pyrophosphate Synthase and Protein Tyrosine Phosphate 1B) in the presence of the acetonitrile, isopropanol and pyrimidine as probes that pMD not only can map competitive binding sites, but also can map allosteric ones. They also demonstrated the need for using the charged probes methylammonium and acetate to capture charged binding sites and other reactive surfaces especially in Protein Tyrosine Phosphate 1B. Prakash and coworkers [32] studied the isolated catalytic domain of G12D K-Ras in the presence of isopropanol as co-solvent in all-atom explicit solvent MD simulations. They have shown that the analysis of these pMD simulations was able to identify all known allosteric pockets of K-Ras.

3. PROBE AGGREGATION AND PROTEIN STABILITY PROBLEMS

Probes used in pMD simulations usually have low molecular weight (below 100 Da). They represent a large subset of functional groups of larger FDA-approved and experimental drugs. They are also fast diffusers, allowing for efficient sampling of the protein surface in short timescales comparable to that of water molecule diffusion. In practice, binding of small low-affinity molecules requires effective concentration of 1M or higher. This is a major problem in pMD simulations as it leads to the aggregation of some co-solvents when used at such high concentration sufficient to saturate binding sites. Furthermore, this aggregation process considerably reduces the effective concentration of the co-solvent, making it more challenging to sample whole protein surface. The aggregation phenomena has been observed previously in pure co-solvent simulations [65] and pMD simulations [35, 49]. Therefore, most of probe molecules used in pMD simulations are chosen to be water soluble. However, using completely hydrophobic probes such as isobutane or benzene could assist in detecting hydrophobic pockets that

might not be exposed in a pure aqueous environment. The modification of Lennard-Jones interactions among probe molecules is demonstrated to overcome this aggregation problem and extend pMD simulations to use aggregation prone molecules [35, 48, 49].

Another issue that is well-known that protein stability is effected by the presence of co-solvents [66-68]. To avoid such protein stability or even denaturation problems, it has been suggested that pMD should be relatively short [33] or harmonic restrains should be added to the protein backbone [61]. However, unrestrained longer pMD simulations have been reported for which no stability issues has been observed [32, 48, 49]. Ultimately, monitoring of protein structure in pMD simulations should always be done to avoid detecting ghost binding sites that only appeared in a partially denatured protein.

4. IDENTIFICATION OF LIGAND BINDING SITES FOR MEMBRANE PROTEINS

Many important drug targets require membrane binding for their biological activity [69-71]. However, it is found that small organic molecules such as the ones used in pMD simulations partition into the hydrophobic core of membranes and lipid aggregates [72-75]. Hence, the presence of small organic molecules appreciably alters the interactions and dynamics of membrane-bound proteins. This seriously limited the usage of conventional pMD methods in studying membrane bound protein targets. Prakash and coworkers confirmed the drastic effects of co-solvent on the structure and dynamics of both the protein and membrane for the membrane-bound K-Ras proteins. They addressed this shortcoming of pMD methods by extending their applicability to membrane-bound drug targets in a way that reduce possible effects of the probe molecules on membrane structure and dynamics [48]. In their method, termed pMD-membrane, isopropanol probe molecules are prevented from membrane partitioning through the modification of selected vdW interactions between the probe and lipid molecules. This approach enabled known allosteric ligand binding site identification on the membrane-bound K-Ras proteins without altering the structure and dynamics of the lipid bilayer and protein. Importantly, pMD-membrane was demonstrated to capture variations in probe molecule surface accessibility as a result of conformational changes due to mutation or membrane binding. Prakash and coworkers also observed that the active site of G12D and G13D K-Ras are different, while G12D and wild K-Ras are similar. Namely, in G13D K-Ras switch I is open and key residues such as Tyr32 have reoriented. They also found differences in probe accessibility to helices 2, 3, and 4 of the two mutants. Despite the fact that these regions are far away from the site of the mutation. They observed that part of the surface of helix 2 where known ligand binding pocket is located was more probe-accessible in G13D than G12D. Similarly, the shallow groove between helices 3 and 4 was also found to be more probe-accessible in G12D than G13D, suggesting that it might be involved in some other important protein-protein interactions.

5. PROBE DENSITY MAPPING TECHNIQUES

Spatial grid-based probe occupancy mappings have been used to quantify probe densities derived from pMD simulations [35]. Hotspot regions are then captured by visualizing iso-surfaces corresponding to high probe occupancy. In other analysis approaches, probe densities are reprocessed and lumped into spheres that are color coded according to corresponding probe-protein estimated local binding free energies [32, 36]. However, these analysis techniques produce spurious probe occupancy regions that are challenging to determine if they represent pocket–like sites without prior knowledge of binding sites of the protein. In general, a binding site occupancy can be affected by simulation time, probe affinity to a binding site and its bulk concentration. Its saturation curve will depend on whether multiple different probes or single probe is used. Furthermore, high bulk probe concentration not only leads to saturation of the whole surface of the protein, but also increases the signal to noise ratio making it difficult to discriminate among hotspots. Graham and coworkers [52] developed MixMD Probeview as a plugin for PyMOL to overcome manual inspection needed to identify relevant binding sites. They reported that Probeview robustly captured known active and allosteric sites by identifying and ranking all binding site total probe occupancy and local probe occupancy maxima for each probe type as derived from a pMD trajectory of a target and probe molecules. Sayyed-Ahmad and Gorfe [49] developed an approach that combines information obtained from a two-dimensional projection technique for globally quantifying probe densities on protein surface and protein surface topology maps derived from probe-binding propensities to protein surface residues. These maps are constructed using a projection technique for globally quantifying probe densities on the protein surface. In addition, surface topography map was shown to reveal protein surface patterns and specific geometrical features. The combined information of both maps is also shown to filter out high probe density hot spots with no pocket-like geometrical characteristics located on flat surface regions or protrusions. This approach was demonstrated to filter out spurious hotspots and discriminates between known K-Ras ligand binding sites and other reactive surfaces.

6. BINDING SITE PHYSICO-CHEMICAL CHARACTERIZATION

To fully characterize a binding site using pMD methods, multiple simulations with one or more of a number of chemically diverse small organic molecules are required. These molecules should have a range of hydrophobic, charged, polar and other chemical characteristics due to the presence of methyl, amide, sulfinyl, carboxyl, hydroxyl and other crucial functional groups. It has been shown that the physico-chemical properties of co-solvents used for pMD simulations affect the detection of ligand binding sites [61]. Therefore, a diverse selection of probe types improves the detectability of ligand binding sites and helps to characterize the local chemical signature by determining the specific interactions and functional groups involved in each probe-protein interaction region. Generally, in the absence of uniquely charged binding sites, both single probe and mixed

probe pMD simulations capture the majority of known pockets [49]. By contrast, using multiple probes including charged ones is needed when charged binding sites are considered, as have been demonstrated for example in the charged probes methyl-ammonium and acetate to capture charged binding sites and other reactive surfaces in Protein Tyrosine Phosphate 1B [51].

7. MAPPING OTHER REACTIVE SURFACES

Hotspot probe regions captured by pMD simulations are not limited to ligand binding site identification. In addition, binding sites that may be involved in protein-protein or protein–membrane interaction can be predicted. It has been demonstrated that pMD simulations can capture dimerization, lipid reactive and crystal packing interfaces. Prakash *et al* [76] suggested that solvent-exposed probe densities observed on the K-Ras surfaces and did not overlap with known ligand binding sites might represent potential dimerization interfaces or as has been suggested later by Sarkar-Banerjee and coworkers as higher order oligomerization interfaces [77]. Similarly, Ung and coworkers [50] showed that pMD simulations can be used to map additional important regions on a protein surface. In particular, these used pMD simulations to map two additional hot spots on the surface of HIV protease (HIVp): the Exo site (between the Gly16-Gly17 and Cys67-Gly68 loops) and the Face site (between Glu21-Ala22 and Val84-Ile85 loops). The Exo site was observed to overlap with crystallographic additives such as acetate and dimethyl sulfoxide that are present in different crystal structures of the protein. Analysis of crystal structures of HIVp in different symmetry groups has shown that some surface sites are interfaces for crystal contacts.

CONCLUSION AND FUTURE OUTLOOK

The utility of pMD methods has been demonstrated to robustly capture known active and allosteric ligand binding sites as well as other reactive surfaces in numerous soluble protein targets. In addition, recent advances in these methods allowed their usage for membrane proteins. They also have the advantage of using physics first principles and incorporate atomic-level solvation effects and protein mobility. Therefore, they are emerging as the method of choice in the identification of ligand binding sites.

A notable drawback of pMD studies reported in the literature is the usage of all-atom additive force fields that inadequately account for electrostatic interactions and molecular polarizabilities. This might lead to inaccuracies in the quantification of probe-target propensities, and hence identification of binding sites. It remains to be shown whether the usage of the more general polarizable force fields can yield different insights. Another problem in most MD simulations is the use of only water soluble probe molecules at high concentrations. This limits the identification of binding sites that are sampled at significantly slower time scales as longer simulations increase the risk of denaturation of the target protein due to interactions with probes. An attractive alternative to overcome this hurdle is using accelerated or high throughput MD simulation with drastically lower probe mole concentrations.

Finally, pMD methods have been applied to a limited number of targets, therefore there is a great opportunity of utilizing it to discovering novel binding sites for many other targets. Furthermore, they have a great promise in the quantification of changes in potential druggability of binding sites of closely related target protein mutations that are linked to different diseases. They also have great protentional in investigating protein-membrane binding poses and protein oligomerization interfaces.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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