Physical Accuracy Leads to Biological Relevance: Best Practices For Simulating Ligand-Gated Ion Channels Interacting With General Anesthetics

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Abstract

Efforts to detect binding modes of general anesthetics (GAs) for pentameric ligand-gated ion channels (pLGICs) are often complicated by a large number of indicated sites, as well as the challenges of ranking sites by affinity and determining which sites are occupied at clinical concentrations. Physics-based computational methods offer a powerful route for determining affinities of ligands to isolated binding sites, but preserving accuracy is essential. This chapter describes a step-by-step approach to multiple methods for identifying candidate sites and quantifying binding affinities, and also discusses limitations and common pitfalls.

1 Introduction

Pentameric ligand-gated ion channels (pLGICs) are widely-studied anesthetic targets, but present numerous challenges for structural characterization even in the apo state. EC_{50} of general anesthetics such as sevoflurane and propofol for pLGICs such as the GABA(A) receptor fall in the 100 mM to 1 μM range, and these low to moderate affinities introduce a high rate of false positives into most approaches. The ability to isolate binding sites offers an advantage to computational approaches, but one of the most common computational approaches, automated docking, is particularly unreliable on its own for interactions of GAs with pLGICs.

We have developed and refined a robust computational approach for identifying candidate binding sites, determining which binding sites are occupied at clinical concentrations, ranking them according to affinity, and determining the microscopic origins

of differences in affinities. This approach considers all atoms of the system explicitly (GA, salt, lipids, water, and protein) and relies on the rigorous physics-based methods of Molecular Dynamics Simulation and Alchemical Free Energy Perturbation.

Our general process essentially involves two components : a screening or discovery phase to identify possible binding modes that might be occupied at clinical concentrations, and a quantification phase in which binding affinities are actually measured. This manuscript describes the steps to set up the necessary calculations and provides examples of the possible pitfalls in this process. This article is written with the CHARMM all-atom force field (1) and the NAMD molecular dynamics software (2)in mind, but the general approach is not forcefield or software specific. In this chapter we focus on a model of an $\alpha_1\beta_3\gamma_2$ GABA(A) receptor in a phosphatidylcholine (POPC) bilayer, interacting with the general anesthetics sevoflurane and propofol. The chapter assumes a general familiarity with the structure of pLGICs as well as the principles underlying classical molecular dynamics simulation.

2 MD simulation involving pLGICs

Setting up MD simulations follows a series of steps that are common for any pentameric channel with/without ligand. The CHARMM-GUI membrane builder website(3), a simulation input generator, accepts a protein structure file as input to embed the protein in a well-packed lipid bilayer with water molecules and neutralizing counter-ions on either side. For a system involving a pLGIC like the GABA(A) receptor, some of the important steps include:

- Choosing membrane builder from the input options.
- Setting up chain names (for non-protein molecules, like the anesthetic, the chain names should match the name mentioned in the topology files)
- Adding terminal patch groups to the protein N-terminus and C-terminus
- Preserving Hydrogen Coordinates
- Adding disulfide bonds.
- Specifying/Preserving protonation states, based on the desired pH of simulation.
- Choosing the right alignment of protein in the membrane. For a pentameric channel, choosing the option to align the 'first principle axis along Z ' would align the channel along Z axis, with the lipid in X-Y plane. Further, aligning the TMD region of protein with lipid involves translocating the protein until the pore center is at the box origin. It is advisable to check the orientation of the channel after this step, by clicking on the 'view structure ' option.
- Choosing the type of lipids for the bilayer and setting water thickness. We typically use the homogenous composition of POPC lipids and choose the default

option of 1.5 layers in CHARMM-membrane builder for choosing the size the lipid membrane. There are $\approx$ 250 lipid molecules in GABA(A) receptor system and a water thickness of 20$\mathring{A}$ is maintained at the top and bottom of the protein.

- Adding salt; we typically use 150mM of neutralizing NaCl.

The total number of atoms is typically between 140,000-200,000.

3 Discovery of Candidate Sites

We use two main approaches for identifying candidate binding modes: 1) Spontaneous Binding of GAs during equilibrium MD, also known as 'flooding' and 2) Docking followed by refinement using equilibrium MD. The former allows a blind, unbiased search for spontaneously occupied binding modes without targeting any particular region of the receptor, but is computationally very expensive, while the latter is primarily suitable when a specific region of the receptor is of interest. We consider each in turn:

3.1 Flooding Simulation

Flooding involves placing a high concentration of GA in the water surrounding the pLGIC (or, in some cases, the membrane) and allowing the GA to spontaneously partition into the membrane and protein binding sites over a simulation time that usually lasts 400 ns - 2μ s. It is most effective for small, relatively soluble ligands, and we have used this approach with isoflurane and nicotinic acetylcholine receptors and the prokaryotic homolog, GLIC(4) as well as sevoflurane interacting with GABA(A) receptors(5). It has also been used with ethanol interacting with glycine receptors(6) and interactions of GAs with voltage-gated channels(7).

Pre-requisites:

- Protein system in a water-box and lipid membrane (Procedure for this is elaborated under the previous section).
- Ligand in PDB format
- Parameters for ligand that are compatible with the forcefield used for water, protein, and lipids (see the chapter by Joseph and Henin in this volume).

Simulation setup :

- Initial Coordinates: Simulation should begin with a receptor embedded in a hydrated lipid bilayer with counter-ions and the GA molecules randomly distributed in the water. GA molecules can be randomly distributed around the receptor in

the desired region of the simulation box using automated tcl scripts and VMD, and then the .pdb file containing both GAs and the receptor can be used as an input into the CHARMM-GUI membrane builder and system can be built as described in section 2.

For a typical simulation box, a single GA molecule in aqueous phase corresponds to a concentration greater than 1 mM. To obtain a range of candidate sites over a reasonable simulation time, at least 20-100 GAs must be present in the simulation. Over the course of a flooding simulation, therefore, the concentration of GA in the aqueous phase may drop from 100mM down to 0-1mM, as GA molecules partition into the lipid phase. The poor correspondence between concentration in the simulation and *in vitro* or *in vivo* systems mandates the use of more sophisticated methods to predict likelihood of occupancy at clinical concentrations, as described subsequently; for a discovery stage, a higher than usual concentration is actually advantageous.

An example of sevoflurane being used to flood the GABA(A) receptor is shown in Figure 7, with a sevoflurane-to-lipid ratio of about 1:3, and a sevoflurane-to-water molecule ratio of about 1:430.

- Non-default NAMD parameters:
  - minimize: This parameter denotes the number of timesteps to minimize the system. Since this simulation involves flooding the system with multiple anesthetic molecules, it is recommended to run longer minimization runs of 50,000 to 100,000 timesteps.
  - constraints: To avoid major changes to the backbone of the protein, this parameter can be set to "on" and a pdb file flagging the backbone atoms to be constrained under the parameters 'conskfile' and 'consref.' Typically the constraints are on the order of 5 kcal/mol/$\mathring{A}$ on the C_α atoms of the protein.

Analysis: Analyzing the trajectory of a flooding simulation using VMD, one could:

- Identify microscopic interactions between protein and anesthetic, including hydrogen bonds as described in 3.3
- Visualize the path of entry of ligand into the binding site.
- Estimate a residence time for a site in which the ligand binds and unbinds several times over the course of the simulation.
- Use VMD plug-in,'VOLMAP tool' to create images showing the average density of the ligand in multiple binding sites (Figure 8 B)
- Visualize the competitive binding between lipid and anesthetic as shown in Figure 8 (A).

- Identify sites likely to have multiple occupancy.
- Identify other binding sites not recognized by docking software. For instance, intra-subunit sites and multiple occupancy of specific sites were identified in flooding simulations with Sevoflurane as shown in Figure 8 (A).

Pitfalls: Some of the possible errors while setting up and running simulation involve:

- Incomplete PSF files being generated after GA is added to the protein, with overlapping the water molecules.
- Insufficient minimization or equilibration. Simulations must be run sufficiently long for GA molecules to partition into the lipid and access binding sites in the protein that may be deep.

Limitations: The high concentrations required can cause aggregation in GAs with poor solubility (such as propofol). It is also usually not practical to calculate concentration dependent occupiancies because of the high concentrations required; AFEP methods described subsequently are essential for estimating features of dose-response.

3.2 Docking

Our approach usually limits use of automated docking to the generation of reasonably favorable binding modes in a particular region of a protein, as initial configurations for significant refinement via MD simulation. Scoring functions from automated docking are rarely meaningful as absolute values, but relative scores may be appropriate for suggesting the most favorable binding mode to use as an initial configuration. Although docking scores are often presented as binding affinities in units of kcal/mol, the method of assigning these units rarely involves an explicit calculation of interaction differences between bound and apo states. Docking scores *cannot* meaningfully be used to calculate a K_D in its usual meaning as the ligand concentration at which half the sites are occupied.

Pre-requisites:

- Receptor model and ligand in PDB format
- Particular region of the receptor of interest, such as the pore, a given subunit interface in the TMD, or the center of a given subunit.

Softwares used:

- Auto-dock Vina (8) is a docking algorithm that predicts a number of preferred orientations of ligand molecule bound to the protein and ranks them based on approximate scoring functions.
- VMD (9) is a software that allows us to visualize and analyze the protein-anesthetic complex following docking.

Docking setup:

- Initial Coordinates: Starting configurations for docking is a PDB structure of the protein and the ligand to be docked with, in the PDB format. In the Figure 2 we have docked sevoflurane and propofol to GABA(A) receptor system.
- Non-default Parameters: Docking can be performed from command-line prompt with a configuration file containing the following commands:
  - flex: Some side-chains of the channel can be made flexible and this part of the protein can be saved as a separate '.pdbqt' file, while the rigid part of the protein would be the argument for the previous command. Use of this command is to facilitate and focus docking in proximity to these 'flex' residues. The software further rotates these 'flex' residues to predict more number of orientations of the ligand at the docked site. This parameter is useful when there is prior knowledge regarding the location of possible binding sites, or specific residues present in the binding site. To select flexible residues, one can name the chain and the residue number into the dialogue box from the toolbar as show in Figure 4B.
  - ligand : Input ligand in '.pdbqt' format. Similar to the receptor, the ligand can be loaded into Autodock tool, in '.pdb' format. Rotatable bonds/torsions must be set for the ligand molecule using the options from toolbar. This specifies the flexibility of the molecule; for propofol all bonds should be left as rigid.
  - center and size : Docking is most suitable when a particular region of the protein has been identified via other means, and this parameter can be used to limit the search space. For instance, the site of interest might be the intersubunit cavity around $\beta N265$, a residue which has been implicated numerous times for propofol ((10–13). (Figure 4). Autodock-tools is useful for visualizing the search space over layed with the protein.
  - exhaustiveness: Docking consists of multiple individual runs starting from random conformations of the protein-ligand complex. The number of these runs depends on the flexibility of the ligand and the protein side-chains and can be controlled by this parameter. We use a value of 15 for this parameter, but also run multiple docking runs with the same parameters for a specific system.
  - nummodes: This parameter specifies the maximum number of binding poses to be generated. We use a value of 20 with multiple individual runs.

Pitfalls: Even with the precautions listed above, the ligand may be inherently unstable in the binding mode for a number of reasons. The two most common are:

- A ligand binding to an unoccupied binding site displaces solvent, and the overall stability depends not just on how the ligand interacts with the protein residues, but

how solvent interacts with the protein, and how the ligand interacts with solvent. Automated docking algorithms do not take into account the latter in a rigorous way even for aqueous solvent. The situation is even worse when the site fills with lipid acyl chains in the absence of ligand, as GA sites on pLGICs often do; in many cases a GA can compete much more favorably with water than with an acyl chain, and docking programs will overestimate the favorability of such a binding mode.

As a result, it is not uncommon for GAs to migrate far enough from a docked location even in shorter ($\sim$ 100 ns long) MD simulations that contact residues are entirely different between the initial and final frame. For example, AutoDock typically returns a binding mode for sevoflurane that is particularly far from the extracellular domain (Figure 6), but in MD simulation, sevoflurane reliably migrates to a site much more similar to that for propofol. Rigorous calculation of affinities using AFEP identifies the latter binding mode as much more favorable than the initial docked conformation.

- The docking algorithm usually uses flexible ligands, with an energy associated with rotation around bonds. Configurations for Propofol in which the hydroxyl clashes with the isopropyl group do not actually have unfavorable dihedral angles and many docking programs will not identify this clash as unfavorable, returning a propofol conformation in which the hydroxyl is overlapping the isopropyl (Figure 5). This can introduce an instability or rapid unbinding in the MD simulation (Figure 3 (A,B)), depending on how equilibration is carried out. For this reason, we strongly recommend removing ligand flexibility when docking with propofol.

3.3 MD Refinement and Characterization of Specific Interactions

Several of the limitations of automated docking can be overcome by refinement with unbiased MD simulation, and if possible, it is almost always advisable to do so before drawing conclusions about microscopic interactions or calculating binding affinity.

Pre-requisites:

- Docked protein-anesthetic complex
- Parameters for the anesthetic.

Simulation Setup:

- Initial Coordinates: Starting configurations for the simulation would be anesthetics docked to binding sites in the protein, generated using docking software.The protein-anesthetic complex can be used as an input to the CHARMM-GUI membrane builder website(3) to build the system.

- Non-default Parameters: restartfreq, dcdfreq, xstFreq, outputEnergies and outputTiming: These parameters denote the number of timesteps between which each output is generated. By default, the configuration files generated by CHARMM membrane builder has these parameters set to 125 or 500 timesteps. A frequency of 5000 timesteps is ideal for further analysis unless a much lower frequency is required.

Analysis: Running the simulation for a considerable about of time ($\approx$ 200-500ns) , one has to analyze the dynamics before setting up FEP simulations. Initial analysis include:

- Checking the stability/mobility of the ligand in the binding site. This can be done by tracking the location of the center of mass (COM) of the ligand in the site throughout the trajectory, using tcl scripts in VMD. Dispersion in the COM is essential to estimate prior to constructing spherical restraints for the ligand while setting up FEP calculations.

- Visualizing microscopic interaction between ligand and its environment. For example, on loading the trajectory into VMD, one can make following selections for a ligand with resname PFL:

  - "(protein and within 3 of (resname SEV)) or (resname PFL)". Making the representation as H-Bonds would allow us to view the hydrogen bonds formed between protein and the ligand (Figure 9).
  - "(protein or lipid or water) and same residue as within 4 of (resname PFL)". This selection would let us see the interactions among the protein residues, water, lipids and the anesthetic, in the binding site. Analyzing these interactions may provide insight into the affinity (obtained through AFEP) of the anesthetic to a specific site, as in (14).

- Identifying higher affinity sites, compared to the sites identified through docking. In the case of sevoflurane docked to a GABA(A) receptor, the low specificity and smaller size of the anesthetic results in greater mobility in the binding site, thus allowing it to explore/move to higher affinity sites as shown in Figure 6.

- Ensure the bound anesthetic is in its favorable/low-energy conformation. In case of docked to GABA(A) system, docking with an unfavorable conformation of propofol (Figure 5) led to immediate expulsion of the molecule as the simulation began. This is described by depicting few snap-shots from the simulation in Figure 3, and this issue can be overcome by removing propofol flexibility during the docking process.

Pitfalls: Some of the common pitfalls encountered during setting up a simulation are:

- Not adding necessary disulphide bonds while setting up the system in CHARMM-GUI.

- Inaccurate protonation states of amino acids at pH 7.

- Not confirming the proper alignment of the protein in the lipid membrane.
- Mismatch in names of the GA atoms in the pdb file and topology file.
- Ligand should have a separate chain name different from the rest of the protein, to be recognized as HETATM by CHARMM-GUI.

Limitations: Inability to witness lipid mixing or domain formation around the receptor in a mixed membrane. This would require extensively long simulations or can be achieved thorough coarse-graining simulations.

4 Calculation of Absolute and Relative Binding Affinities

Following the identification of a stable binding mode for the anesthetic, the binding affinity can be rigorously calculated using a theoretically exact simulation technique called Alchemical Free Energy Perturbation (AFEP) that relies on the equation introduced by Zwanzig(15) for calculating the difference in Helmholtz free energy between two states, X and Y:

$$\Delta A_{X\rightarrow Y} = -RT \ln \left\langle e^{-(H_Y(\mathbf{r})-H_X(\mathbf{r}))/RT} \right\rangle_X \tag{1}$$

where R is the gas constant, T is temperature, $H(\mathbf{r})$ is the Hamiltonian for state X or state Y. In a binding free energy application, X would represent the bound state, Y would represent the apo state, and $\Delta A_{X\rightarrow Y} \sim \Delta G_{X\rightarrow Y}$ because ligand binding will not significantly change the system volume. In practice (16, 17) for convergence purposes this is carried out by summing over a series of windows in which the interaction between the ligand and all other atoms of its environment are gradually decreased to zero:

$$H_{\lambda_i} = H_{\text{env}} + H_{\text{lig}} + (1-\lambda_i)H_{\text{env-lig}}, \tag{2}$$

where $\lambda = 0$ is equivalent to the bound state X, $\lambda = 1$ is the unbound state Y, $0 <= \lambda_i < 1$ for all i and $\lambda_i < \lambda_{i+1}$. The free energy change for "decoupling" the ligand from its environment is

$$\Delta G_{\text{env}} = -RT \sum_i \ln \left\langle e^{-(H_{\lambda_{i+1}}(\mathbf{r})-H_{\lambda_i}(\mathbf{r}))/RT} \right\rangle_{\lambda_i} = -RT \sum_i \ln \left\langle e^{-(\lambda_i-\lambda_{i+1})H_{\text{env-lig}}/RT} \right\rangle_{\lambda_i}. \tag{3}$$

Treating the coupled ligand as the bound state requires that the fully coupled ensemble (at $\lambda = 0$) includes no states with ligand outside the binding site. This assumption can breakdown for moderate affinity ligands like GAs, but can be resolved by making the requirement of localization to the binding site explicit, via a restraint potential on the ligand center of mass.

A flat-well potential that vanishes within the binding site but is very high outside the binding site accomplishes this with minimal need for correction, and also alleviates

convergence problems for windows close to $\lambda = 1$ (fully decoupled). The standard binding affinity is

$$\Delta G^0 = \Delta G_{\text{site}} - \Delta G_{\text{solv}} - k_B T \ln(V_{site}/V^0) \quad (4)$$

where ΔG_{site} is free energy of decoupling from environment of the protein binding site, ΔG_{bulk} is the free energy of decoupling ligand from bulk solvent(solvation free energy), V_{site} is the volume accessible to the center of mass of the bound ligand (as specified by Eq. 5), and $V^0 = 1661\text{Å}^3$ is the accessible volume per molecule in a 1M solution. Although this method is theoretically exact and all degrees of freedom are considered automatically by the method, the success and inherent challenge of the technique relies on obtaining reasonable convergence of each average in Equation 3.

Pre-requisites:

- Starting configuration from MD simulation.
- PDB files indicating the atoms/ligands to be unbound/decoupled.
- Restraint files indicating the type of restraints to be applied to the movement of the ligand during the simulation.
- Solvation free energy of the anesthetic. This involves, decoupling the ligand from bulk solvent in the absence of protein. This is required to calculate the free energy cost of moving the ligand from bulk solvent to a binding site in the protein.

Simulation Setup :

- Initial coordinates: Starting coordinates should be taken from the output of a fully interacting equilibrium MD simulation, as described in the previous section.
- Non-default parameters:
  - alchEquilSteps : the number of steps at the beginning of each alchemical window that is excluded in the cumulative average used to calculate $\Delta\Delta G_i \equiv -RT \ln \left\langle e^{-(\lambda_i - \lambda_{i+1})H_{\text{env-lig}}/RT} \right\rangle_{\lambda_i}$ for window i, to allow the system to adjust to the new value of λ. We typically use values of 25000 to 50000 steps to balance the need for equilibration time with need for actual samples. It affects on-the-fly analysis but not the actual trajectory, and different values can be used in post-processing.
  - alchElecLambdaStart : the value of λ for which the electrostatics should be entirely switched off, through the soft-core potential necessary for FEP calculations. We use the value of 0.5 recommended by the NAMD User's guide (18).
  - alchVdwLambdaEnd : the value of λ for which the Van der Waals interaction should be entirely switched off, through the soft-core potential necessary for FEP calculations. We use the value of 1.0 recommended by the NAMD User's guide (18).

  - alchDecouple : This parameter specifies whether either intermolecular interactions of the anesthetic are turned 'off' (decoupling) or both inter and intra-molecular interactions are turned 'off' (annihilation). By default this parameter is set to 'off' which chooses the annihilation option. We use the decoupling method, i.e, set the parameter 'on' as the intramolecular annihilation free energies simply get canceled when the solvation free energy is subtracted.
  - alchLambda/alchLambda2 : Every configuration file would have different values for λ, denoting the progress of the perturbation. We typically use windows with $\lambda_{i+1} - \lambda_i = 0.05$ for λ between 0 and 0.8 and $\lambda_{i+1} - \lambda_i = 0.025$ for λ between 0.8 and 1.0. These values can be modified during re-runs to improve sampling.

- Additional files:

  - Restraint file: We use flat-bottom spherical restraints for the inter and intra-subunit sites with the following potential:

$$U_{rest}(\vec{r}_{COM}) = \begin{cases} \frac{k(\vec{r}_{COM}-R)^2}{2}, & |\vec{r_0} - r_{COM}| > R \\ 0, & |\vec{r}_0 - r_{COM}| <= R \end{cases} \tag{5}$$

    where $\vec{r}_{COM}$ is the ligand center of mass, $\vec{r}_0 = \langle \vec{r}_{COM} \rangle$ in the fully coupled and bound state, and $R = \max(|\vec{r}_0 - r_{COM}|)$ in the fully coupled and bound state. Both $\vec{r}_0$ and R can be determined from Equilibrium MD simulations, and determine the value $V_{site} = 4/3\pi R^3$.

    Typical values of k are 5 kcal/mol/Å. For pore sites we adjust Equation 5 to represent a cylindrical site by applying separate potentials on the vertical and radial coordinates. In NAMD, these restraints can be implemented using tclForces or the collective variables module, both of which require an additional file. For simple geometries, using tclForces tends to be computationally faster and is implemented using the parameter 'tclForces' in the configuration file.
  - FEP file : PDB file used to denote the atoms that are to be decoupled. Can be generated using VMD, where the 'beta' column in the PDB file can be flagged with number '-1.00' for outgoing atoms and '1.00' for incoming atoms.

Analysis:

- Multiple FEP runs with same or different starting configurations can be performed to check the consistency of the results.
- Performing a recoupling run(ligand-unbound state to bound state) followed by decoupling and combining the results using BAR or SOS estimator to obtain more accurate binding energy value.

- Understanding the affinity values obtained. The standard free energy of binding is related to the dissociation constant K_D via,

$$\Delta G^0 = RT \ln K_D \tag{6}$$

  Any K_D value that is smaller (stronger) than the EC_{50} of a GA indicates the site may be essential to action of the GA. Previously we used measured K_D for isoflurane in the GLIC pore to argue that inhibition by isoflurane likely occurred via pore block rather than an allosteric mechanism;(19) although there is still no available crystal structure for isoflurane bound to GLIC, crystal structures released several years later confirmed pore block as the dominant mechanism for a homologous prokaryotic channel, ELIC(20). Similarly, we were able to rank K_D for propofol interacting with $GABA_Ar$ subunit interfaces, with sites containing α and β subunits having K_D <EC_{50} but sites with γ subunits having $K_D > EC_{50}$, consistent with results using photolabeling with azipropofol and a click agent in neurons. Correlating these results with observations of interactions from Equilibrium MD explained the surprising result that propofol had a particularly low affinity for the site with the most polar residues, due to competition with water.(14)

Pitfalls:

- Insufficient sampling to properly calculate the necessary averages is a primary pitfall (21); it can be assessed by examining $\Delta\Delta G$ per window by plotting the last column of the '.fepout' output file from the FEP simulation (Figure 11). Converged windows will flatten out by the end of the window, while abrupt changes indicate a need for:
  - Extending the calculation for that window.
  - Further dividing $\delta\lambda$ into multiple separate windows.
  - Removing some of the early values from the average (equivalent to increasing alchEquilSteps)
- Insufficient total simulation time to sufficiently equilibrate decoupled states. One way to assess this is tracking the rehydration of the binding site following the decoupling of the ligand, by comparing the number of solvent molecules with a hydrated site in an apo simulation. Increasing the overall simulation time is necessary if the site does not become fully solvated, and usually adding this time to later, mostly decoupled windows works best.
- Neglecting the analytical correction for the standard state in Equation 4.
- Applying restraints with inaccurate values for R or $\vec{r}_0$. Analyzing unbiased MD simulations of the protein-ligand complex for each potential site is critical for doing this properly.

- Beginning AFEP using a configuration generated by docking without performing sufficient equilibration. In the case of Sevoflurane docked to a lower site in the TMD (Figure 6 A), FEP run resulted in an affinity in the 0.1 M range, while AFEP runs with well equilibrated conformations (Figure 6 B) yielded affinities in the 20-200mM range, on the order of EC_{50}.

Summary

We have presented a step-by-step approach to identifying candidate binding sites and quantifying and ranking affinities of volatile and injected general anesthetics with pentameric ligand-gated ion channels. While automated docking calculations do have limited usefulness, the realistic parameterization and explicit water and lipids used in Molecular Dynamics simulation is particularly important for characterizing interactions of GAs with binding sites in pLGICs. For identifying multiple candidate sites, flooding simulations provide a useful balance between classical simulation that is straightforward to interpret and conceptualize in analogy with experiments, while still maintaining a realistic environment and set of interactions. Alchemical free energy perturbation calculations can provide high accuracy estimates for affinities if carried out carefully, but require skill and careful attention to convergence.

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Figure 1: Flowchart describing the sequential steps involved in performing (left) Flooding simulation and (right) Docking using Autodock and MD simulation

Figure 2: View of the TM domain, looking down on the membrane from the extracellular region; Sevoflurane(A) and Propofol(B) docked to the TMD of GABA(A) receptor. Docking was individually performed at all the inter-subunit sites and the pore by making few of the protein residues, flexible. For instance, at the α-β site, βMET289 residue side-chain was made flexible while docking to this interface.

Figure 3: View of the TM domain, looking down on the membrane from the extracellular region; MD of PFL-GABA(A) receptor system, images depict the position of Propofol at initial and final frames of the trajectory. Unfavorable conformation of Propofol led to expulsion of the ligand in the course of the simulation.

Figure 4: Screenshot of Autodock tools screen depicting a dialogue box describing (A) the measurements of the grid box over the TMD of the protein and (B) the process of selecting the flexible residues in the protein.

Figure 5: Comparison between an unfavorable (A) and favorable low energy (B) conformation of Propofol.

Figure 6: Side view of the sevoflurane in the β-α interface of the GABA(A) receptor system; Two images depicting the docked (A) and equilibrated conformation (B) of the system. Equilibration of the docked conformation allows Sevoflurane to re-orient itself at a higher affinity site.

Figure 7: Cross-sectional view of the Sevoflurane flooded GABA(A) receptor system with the TMD aligned along POPC lipid membrane(colored by name) and placed in a box of explicit water (molecules not shown, represented as a blue box); (A) Initial frame with Sevoflurane(colored by name) flooded in the water; After 1.5μs Sevoflurane completely localizes in the lipid membrane with some binding the inter and intra-subunit sites(red and orange) in the TMD.

Figure 8: View from the extracellular domain, of a Sevoflurane flooded GABA(A) receptor system; (A) Final frame from the flooding simulation showing sevoflurane occupying the inter, intra and pore sites(Red, orange) overlaid with sevoflurane(yellow) docked using Auto-dock; Flooding simulation also identified lipid (colored by name) interference in β intra-subunit site and and α-γ, α-β intersubunit sites. (B) Image created using VMD plugin, VOL-MAP tool, to depict the density isosurface (orange) averaged over the last 700 ns of the simulation; large mesh represent occupation over majority of the trajecotory, whereas a few smaller mesh represents occupation for lesser time.

Figure 9: Depiction of Propofol interacting with water and protein side-chains at the $\beta-$-$\alpha+$ intersubunit site, in a snapshot from a MD simulation.

Figure 10: Flowchart describing the sequential steps involved in calculating binding affinity of anesthetics

Figure 11: Sample data set for $\Delta\Delta G_i$ variations per window.Curves that plateau (as at $\lambda = 0.825$ to 0.85) indicate convergence, while curves that still change rapidly by the end of the window (as at $\lambda = 0.20$ to 0.25) indicate a need for extending the calculation for that window or dividing the window into two.