The effect of the multiple mutations in Omicron RBD on its binding to human ACE2 receptor and immune evasion: an investigation of molecular dynamics simulations

Leyun Wu\textsuperscript{1,4,\#}, Liping Zhou\textsuperscript{1,4,\#}, Mengxia Mo\textsuperscript{2,\#}, Yishui Li\textsuperscript{2}, Jiaxin Han\textsuperscript{1}, Jintian Li\textsuperscript{1,4}, Yanqing Yang\textsuperscript{1,4}, Xinben Zhang\textsuperscript{1}, Chunye Gong\textsuperscript{2}, Kai Lu\textsuperscript{2}, Likun Gong\textsuperscript{3,4}, Chengkun Wu\textsuperscript{2,*}, Weiliang Zhu\textsuperscript{1,4,*}, and Zhijian Xu\textsuperscript{1,4,*}

\textsuperscript{1}CAS Key Laboratory of Receptor Research; Drug Discovery and Design Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
\textsuperscript{2}College of Computer Science and Technology, National University of Defense Technology, Changsha 410073, China
\textsuperscript{3}Center for Drug Safety Evaluation and Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
\textsuperscript{4}School of Pharmacy, University of Chinese Academy of Sciences, Beijing 100049, China

\#Leyun Wu, Liping Zhou and Mengxia Mo contributed equally to this work.

*To whom correspondence should be addressed.

E-mail: chengkun_wu@nudt.edu.cn (C.W.), wlzhu@simm.ac.cn (W.Z.), zjxu@simm.ac.cn (Z.X.).

ORCID: 0000-0002-9688-5311 (C.W.), Weiliang Zhu: 0000-0001-6699-5299 (W.Z.), Zhijian Xu: 0000-0002-3063-8473 (Z.X.).
Abstract

SARS-coronavirus-2 (SARS-CoV2) Omicron variant (B.1.1.529) is of great concern to the world due to multiple mutations that may have an impact on transmissibility and immune evasion. Compared to the wild type (WT), there are 15 mutations in the Omicron receptor-binding domain (RBD), 10 of which are in the receptor-binding motif (RBM), where the host angiotensin-converting enzyme 2 (ACE2) interacts directly with. As a comparison, the currently dominant variant Delta (B.1.617.2) only has 2 mutations (L452R and T478K) or an additional E484K mutation in the RBM. As many as 15 mutations in Omicron RBD make it very hard to predict whether the mutations would increase the binding affinity to ACE2, particularly considering that 10 mutations crowded in the RBM. To understand the combinatorial mutation effect on Omicron RBD binding to ACE2 and potential immune evasion, we calculated the binding affinities of the WT/Delta/Omicron RBDs to ACE2 and antibodies with 600 ns molecular dynamics simulations for each system. We found that Omicron RBD has slightly weaker ACE2 affinities than WT RBD (−29.39 ± 2.96 Kcal/mol vs. −33.13 ± 3.26 Kcal/mol), and much lower affinities than Delta RBD (−42.76 ± 2.38 Kcal/mol). Further analysis revealed that Omicron N501Y increase ACE2 binding but Q493K and Q498R decrease ACE2 binding. In addition, Omicron RBD might escape the launched monoclonal antibodies (mAbs) Etesevimab and clinical BD-368-2 but may still sensitive to the launched mAbs Bebtelovimab.

Introduction

Highly contagious coronavirus disease 2019 (COVID-19) is caused by SARS-coronavirus-2 (SARS-CoV2). Since the start of the epidemic outbreak, several Variants of Concern (VOC), e.g., Alpha, Beta, Gamma, Delta and Omicron, have emerged [1]. The current global epidemiology of SARS-CoV-2 is characterized by a predominance of the Delta variant. Of 839 119 sequences uploaded to GISAID database with specimens collected in the last two months, more than 99.8% are Delta variant[2]. Liu et al. found that the binding affinity between R452/K478-RBD of Delta (B.1.617)
variant and ACE2 is ~2 times higher than that of wild type (WT) RBD and ACE2 (8.3 nM)[3]. Therefore, the viral mutation may have high risk of increasing transmissibility and virulence or decreasing of effectiveness of therapeutics, making it more difficult to control the epidemic situation effectively.

SARS-CoV-2 variant Omicron (B.1.1.529) was newly announced by the World Health Organization (WHO) on November 26, 2021. Astonishingly, this variant carries as many as 30 single point mutations, 3 deletion mutation and one insertion mutation on spike protein, the main antigenic target of many monoclonal antibodies[4]. Among the mutations, 15 of them are on receptor-binding domain (RBD), especially 10 mutations are in the receptor-binding motif (RBM), which can interact directly with the angiotensin-converting enzyme 2 (ACE2) entry receptor on host membranes when the virus infects host cells[5]. In comparison, other detrimental variants have less than 5 RBD mutations[6]. For example, Delta (B.1.617.2) only has 2 mutations (L452R and T478K) or an additional E484K mutation in the RBM. Therefore, it is speculated that Omicron variant may significantly impact the effectiveness of current prophylactic and/or therapeutic drugs and binding affinity to ACE2. Consequently, Omicron mutant has aroused wide concern and many countries have taken measures on entry restrictions to prevent its rapid spread.

Bloom et al. systematically changed every single amino acid in the RBD of the spike protein and determine the effects of the substitutions on ACE2 binding[7]. By checking the 15 Omicron RBD mutations, it was found that 9 single mutations (S371L, S373P, S375F, K417N, G446S, E484A, G496S, Q498R, Y505H) should decrease the binding affinity to ACE2 while 6 mutations (G339D, N440K, S477N, T478K, Q493K, N501Y) might increase the binding affinity to ACE2. However, different from Alpha, Beta, Gamma and Delta variants, Omicron have much more mutations, making the predictions for ACE2 binding and immune evasion very difficult due to the combinatorial mutations, particularly considering that 10 mutations crowded in the RBM interacting with each other.

To understand the combinatorial mutation effect on Omicron RBD binding to ACE2 and potential immune evasion, we calculated the binding affinities of the
WT/Delta/Omicron RBDs to ACE2 and antibodies by 600 ns molecular dynamics (MD) simulations for each system to shed light on the detailed implications of SARS-CoV2 Delta and Omicron variant on COVID-19 epidemiology, severity, effectiveness of public health and social measures, or other relevant characteristics. We found that Omicron RBD has slightly weaker ACE2 affinities than WT RBD (-29.39 ± 2.96 Kcal/mol vs. -33.13 ± 3.26 Kcal/mol), and much lower affinities than Delta RBD (-42.76 ± 2.38 Kcal/mol). Further analysis revealed that Omicron N501Y increase ACE2 binding but Q493K and Q498R decrease ACE2 binding. In addition, Omicron RBD might escape the launched monoclonal antibodies (mAbs) Etesevimab and clinical BD-368-2 but may still sensitive to the launched mAbs Bebtelovimab.

**Methods**

**Preparation of mAb-S protein and ACE2-S protein complexes**

The Omicron spike trimer was modelled by SWISS-MODEL Server in Alignment mode[8]. The Omicron homology model with the RBD up was chosen for further analysis. The Omicron spike were superimposed to a WT spike/ACE2 complex (PDB ID: 6vw1[9]) to create an Omicron-ACE2 complex. We retrieved 3 marketed or clinical RBD-specific antibodies bound to S protein from the Protein Data Bank. The Omicron RBD domain containing residue 334-526 were truncated from the full-length S protein. In order to get the intact structures for WT/Delta RBD and antibodies, missing residues in flexible loops were modeled using SWISS-MODEL. Delta RBD model was created by PyMOL2.5[10] to yield K417N and E484K on the basis of 7vvs[11]. The Delta model in our simulations have 4 mutations: K417N, L452R, T478K, and E484K.

**System preparation**

Protonation states were assessed using H++ 3.2[12, 13] at pH 7.4. A cubic explicit water box described using the TIP3P model was used to solvated the complex system, which was extended by 10 Å from the solute. An atmosphere of 150 mM NaCl was also included in all simulations. The generated models were parametrized using amber ff1+4SB force fields[14] for protein. Subsequently, the parameter files created by tleap
in Amber18[15] were converted to gromacs format. About, 5000 steps of minimization including 2500 steps of steepest descent minimization and 2500 steps of conjugate gradient minimization were performed to remove bad contacts during the energy minimization phase. Equilibration in NPT ensemble was run at 1.0 bar and 300 K for 500 000 steps at 2 fs/step. Gromacs2020.2[16, 17] software package was used to run the minimization, equilibration simulations with position constraints (1 kcal/mol/Å²) on protein.

**Molecular dynamics (MD) simulations**

Mdrun module in Gromacs2020.2 was used to perform 200ns MD production simulations at 300 K, 1 bar for all complexes. Temperature and pressure were controlled by Langevin thermostat[18] and a Nosé-Hoover Langevin barostat[19, 20]. Bonds involving hydrogen atoms were fixed by the SHAKE algorithm[21]. The cutoff distance applied for van der Waals interactions was 10 Å. All simulations were performed using particle-mesh Ewald (PME) for long-range electrostatic interactions[22]. Mdconvert[23] was used to convert the trajectories to amber format. Cpptraj module in Amber18 was used for trajectory processing and analysis.

**Binding free energy calculation**

Binding free energy (ΔG) of RBD-antibody or ACE2-RBD complexes was calculated by MM/GBSA[24] method using GB OBC model (igb = 5) with a salt concentration of 150 mM. 750 snapshots extracted evenly from 50-200ns trajectories were used for binding free energy calculation. In this study, the internal and external dielectric constants were set to be 1.0 and 78.5 separately. The free energy decomposition analysis was carried out using an internal program with idecomp = 1.

**Results and Discussion**

**Hard to instinctively predict the effect of the crowed multiple mutations in Omicron RBD on its binding to human ACE2 receptor and immune evasion**

There are 1273 amino acids in the WT spike (UniProt ID: P0DTC2), which is composed of S1 (residues 13 – 685) and S2 (residues 686 – 1273)/S2' (residues 816 – 1273). The
RBD domain are composed by residues 319-541 and RBM are residues 437-507. Omicron spike is 3 amino acids shorter than the WT spike. There are 30 single point mutations, 3 deletion mutation and one insertion mutation on Omicron spike protein, i.e., A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K and L981F. Because RBD is the most important domain for the ACE2 binding, we will focus on RBD in this study. 15 Omicron RBD mutations, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, and Y505H are shown in Figure 1. As a comparison, Delta RBD only have 4 mutations, K417N, T484K, E484K, and L452R. It could be seen that the Omicron mutations are not evenly distributed in RBD, but crowded (10 residues, i.e., N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H) in RBM, which interacts directly with ACE2. The crowded feature of the Omicron RBD mutations suggests that the mutations are not independent, which means that we could not simply add the 15 single substitution together to get a correct answer. We will put all the 15 Omicron RBD mutations together and observe what will happen in the MD simulations.
Omicron RBD doesn’t enhance binding affinity to ACE2

We performed 200ns all-atom molecular dynamics simulation for WT, Delta and Omicron variant RBD-ACE2 complex in triplicate and the mean value of three repetitions for each measurement was used for analysis. According to the predicted binding free energy (Table 1), the ΔGs of ACE2-RBD<sub>Δelta</sub> in three repeating experiments are all about 10 kcal/mol higher than that of ACE2-RBD<sub>WT</sub>. The average number of ΔG of RBD<sub>Δelta</sub> to ACE2 (-42.76 ± 2.38 kcal/mol) demonstrated stronger binding than that of RBD<sub>WT</sub> (-33.13 ± 3.26 kcal/mol). The result is in good agreement with existing experimental findings, proving that our method is reliable to a certain extent[3]. By contrast, the difference of ΔG between ACE2-RBD<sub>Omicron</sub> and ACE2-RBD<sub>WT</sub> is only 3.74 kcal/mol (Table 1), suggesting that although there are a high number of mutations in S protein RBD, they don’t increase affinity to ACE2 and even slightly weaken the binding.

Table 1. The predicted ACE2-RBD binding free energy (kcal/mol) of WT, Delta and Omicron variants

|                  | Average   | 1         | 2         | 3         |
|------------------|-----------|-----------|-----------|-----------|
| ACE2-RBD<sub>WT</sub> | -33.13 ± 3.26 | -29.83 ± 0.36 | -33.20 ± 0.34 | -36.35 ± 0.30 |
| ACE2-RBD<sub>Δelta</sub> | -42.76 ± 2.38 | -40.49 ± 0.30 | -42.55 ± 0.29 | -45.25 ± 0.25 |
| ACE2-RBD<sub>Omicron</sub> | -29.39 ± 2.96 | -26.37 ± 0.26 | -29.52 ± 0.23 | -32.29 ± 0.27 |

Binding free energy decomposition

In order to understand the detailed implications of every mutation on the binding to ACE2, we conducted binding free energy decomposition based on all nine 50-150ns trajectories. As indicated in Figure 2A, we found that although there were some fluctuations in the three experiments, the energy contributions of residue 498, 493, 505,
and 496 in Omicron variant RBD are smaller than that in WT especially for residue 498. However, the energy contribution of R501 in Omicron variant is greatly larger than N501 in WT. Therefore, the combined effects of the above mutations make the final binding free energy change subtle. Nevertheless, from the change in energy contributions of mutations between WT and Delta variant, there are not huge differences in those four mutations (residue 452, 478, 417, 484). From overall per residue energy decomposition results in the receptor-binding motif (RBM) region (Figure 2B), the binding free energies of F486, Y489, F490 increase in Delta mutant compared with WT. This implies that these residues in Delta variant may contribute more to hydrophobic effects and thus the final affinity to ACE2 is improved.
Figure 2. The binding free energy decomposition of residues in the different RBDs. (A) Energy contributions of 16 RBD residues with mutations in Delta or Omicron variants. (B) Energy contributions of RBM residues (437-507).

The role of N501Y in Omicron variants
The binding free energy decomposition of residues (Figure 2) shows that energy contribution of N501 in RBDWT is \(-2.12 \pm 1.13\) kcal/mol, while the energy contribution of Y501 in RBD\(_{\text{Omicron}}\) is \(-6.97 \pm 0.30\) kcal/mol (a stronger attraction). By analyzing molecular dynamic trajectories, we found that Y501\(_{\text{RBD}}\) and Y41\(_{\text{ACE2}}\) could form a strong π-π stacking interaction (Figure 3A). As shown in Figure 3B, the center of mass distance between Y501\(_{\text{RBD}}\) and Y41\(_{\text{ACE2}}\) is around 5.5 Å throughout the 200ns trajectory.

Figure 3. (A) Diagram of Y501\(_{\text{RBD}}\)-Y41\(_{\text{ACE2}}\) π-π stacking interaction. (B) The distance between the center of mass of Y501\(_{\text{RBD}}\) and Y41\(_{\text{ACE2}}\).

**The role of Q493K and Q498R in Omicron variants**

The common feature of Q493K and Q498R mutation is that both of them mutates from electrically neutral Q to positively charged amino acids (K and R). By binding free energy decomposition, the energy contributions of both K493 and R498 are decreased. Specifically, the energy contribution of K493 in RBD\(_{\text{Omicron}}\) decreased about 1.90 kcal/mol to Q493 in RBD\(_{\text{WT}}\), and the energy contribution of R498 in RBD\(_{\text{Omicron}}\) decreased about 4.15 kcal/mol to Q498 in RBD\(_{\text{WT}}\). As shown in Figure 4, Q493 in RBD\(_{\text{WT}}\) could form tight interactions with K31 and E35 in ACE2. By calculating distances of K31\(_{\text{ACE2}}\)-Q493\(_{\text{RBD}}\) and K31\(_{\text{ACE2}}\)-K493\(_{\text{RBD}}\) (Figure 4A), we found that K31\(_{\text{ACE2}}\) moves away from K493\(_{\text{RBD}}\), which may be due to the repulsion of lysine with the same electrical properties. As shown in Figure 5C, K353\(_{\text{ACE2}}\) also moves away from R498\(_{\text{RBD}}\). Hence, we speculate that the repulsion between positively charged residues is the main cause of the decreased binding free energy in ACE2-RBD\(_{\text{Omicron}}\). Differently, K493\(_{\text{RBD}}\) forms a tighter interaction with E35\(_{\text{ACE2}}\) (Figure 4B), which partly compensates for the effect of positively charge repulsion. While the lack of
compensation effect in R493\textsubscript{RBD} (Figure 5A &5B) leads to its severe decreased interactions with ACE2.

Figure 4. (A) Minimum distance between K31\textsubscript{ACE2} and Q/K493\textsubscript{RBD}. (B) Minimum distance between E35\textsubscript{ACE2} and Q/K493\textsubscript{RBD}.

**Omicron variant may cause immune evasion from some antibodies**

To evaluate the binding affinity between three launched or clinical mAbs and two variant RBD, MM/GBSA calculations were carried out with RBD\textsubscript{WT} as control based on each MD runs lasting 200ns in triplicate. As shown in Table 2, the relative binding free energy to Etesevimab and BD-368-2 between WT and Delta RBD is -25.44, -17.21 kcal/mol, which means this variant may impair the effectiveness of these two antibodies. In comparison, the difference in binding affinity to Bebtelovimab is only -4.81 kcal/mol, suggesting that the potency of this antibody is less likely to be affected by the mutant. Similar to Delta variant, mutations in Omicron variant may have detrimental effect on the efficacy of Etesevimab and BD-368-2, while make little effect on Bebtelovimab.
Table 2. The predicted mAb-RBD binding free energy (kcal/mol) of WT, Delta and Omicron variants

| Antibody | PDB ID | System  | Average  | 1         | 2         | 3         |
|----------|--------|---------|----------|-----------|-----------|-----------|
| Etesevimab CB-6 (Launched) | 7C01 | RBD<sub>WT</sub> | -67.78 ± 2.12 | -65.33 ± 0.34 | -68.85 ± 0.33 | -69.15 ± 0.34 |
|          |        | RBD<sub>Delta</sub> | -42.34 ± 5.78 | -37.64 ± 0.29 | -40.6 ± 0.33  | -48.79 ± 0.34  |
|          |        | RBD<sub>Omicron</sub> | -39.75 ± 1.63 | -36.28 ± 0.43 | -38.04 ± 0.45 | -39.54 ± 0.39  |
| Bebtelovimab LY-CoV-1404 (Launched) | 7MMO | RBD<sub>WT</sub> | -58.77 ± 2.97 | -56.52 ± 0.32 | -57.66 ± 0.40 | -62.14 ± 0.58  |
|          |        | RBD<sub>Delta</sub> | -53.96 ± 2.27 | -51.49 ± 0.28 | -54.45 ± 0.26 | -55.95 ± 0.34  |
|          |        | RBD<sub>Omicron</sub> | -59.09 ± 4.37 | -54.76 ± 0.34 | -59.02 ± 0.28 | -63.50 ± 0.35  |
| BD-368-2 (Clinical) | 7CHH | RBD<sub>WT</sub> | -28.30 ± 5.96 | -22.42 ± 0.32 | -28.14 ± 0.33 | -34.33 ± 0.26  |
|          |        | RBD<sub>Delta</sub> | -11.09 ± 5.86 | -7.08 ± 0.24  | -8.37 ± 0.26  | -17.82 ± 0.33  |
|          |        | RBD<sub>Omicron</sub> | -13.31 ± 6.81 | -7.64 ± 0.21  | -11.42 ± 0.40 | -20.86 ± 0.29  |

Figure 5. (A) Minimum distance between K31<sub>ACE2</sub> and Q/K493<sub>RBD</sub>. (B) Minimum distance between E35<sub>ACE2</sub> and Q/K493<sub>RBD</sub>. 
Conclusion

It’s very hard to instinctively predict the effect of the crowded multiple mutations in Omicron RBD on its binding to human ACE2 receptor and immune evasion. Utilizing molecular dynamics which put all 15 Omicron RBD mutations together, we found that Omicron RBD has slightly weaker ACE2 affinities than WT RBD (-29.39 ± 2.96 Kcal/mol vs. -33.13 ± 3.26 Kcal/mol), and much lower affinities than Delta RBD (-29.39 ± 2.96 Kcal/mol vs. -42.76 ± 2.38 Kcal/mol). Further analysis revealed that Omicron N501Y increase ACE2 binding but Q493K and Q498R decrease ACE2 binding. However, we should point out that weaker Omicron RBD binding to the ACE2 receptor does not mean that Omicron have a lower transmissibility. Omicron H655Y and N679K, proximal to the furin cleavage site, might increase spike cleavage and make the virus more contagious. Omicron P681H could increase spike cleavage to increase transmissibility. In addition, spike trimer may function differently from a single RBD. Lastly, we calculate the binding energies between Omicron/WT/Delta RBD and mAbs and found that Omicron RBD might escape the launched Etesevimab and clinical BD-368-2 but may still sensitive to the launched Bebtelovimab.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Acknowledgments

This work was supported by the Natural Science Foundation of Shanghai (21ZR1475600) and National Key R&D Program of China (2016YFA0502301 & 2017YFB0202601), the National Science Foundation of China (U1811462) and the open fund from the State Key Laboratory of High-Performance Computing (No. 201901-11). The work was partially carried out at National Supercomputer Center in Tianjin, and the calculations were performed on the new generation Tianhe
References

1. Cavallo G, Metrangolo P, Pilati T, Resnati G, Sansotera M, Terraneo G. Halogen bonding: a general route in anion recognition and coordination, *Chem Soc Rev* 2010;39:3772-3783.

2. Weekly epidemiological update on COVID-19-30 November 2021. [https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---30-november-2021](https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---30-november-2021) (30 November 2021, date last accessed).

3. Liu H, Wei P, Zhang Q, Aviszus K, Linderberger J, Yang J, Liu J, Chen Z, Waheed H, Reynoso L, et al. The Lambda variant of SARS-CoV-2 has a better chance than the Delta variant to escape vaccines, *bioRxiv: the preprint server for biology* 2021:2021.2008.2025.457692.

4. European Centre for Disease Prevention and Control. Implications of the emergence and spread of the SARS-CoV-2 B.1.1.529 variant of concern (Omicron), for the EU/EEA. 26 November 2021. ECDC: Stockholm; 2021.

5. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor Recognition by the Novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus, *J Virol* 2020;94.

6. Mohammadi M, Shayestehpour M, Mirzaei H. The impact of spike mutated variants of SARS-CoV2 [Alpha, Beta, Gamma, Delta, and Lambda] on the efficacy of subunit recombinant vaccines, *The Brazilian Journal of Infectious Diseases* 2021;25:101606.

7. Starr TN, Greaney AJ, Hilton SK, Ellis D, Crawford KHD, Dingens AS, Navarro MJ, Bowen JE, Tortorici MA, Walls AC, et al. Deep Mutational Scanning of SARS-CoV-2 Receptor Binding Domain Reveals Constraints on Folding and ACE2 Binding, *Cell* 2020;182:1295-1310 e1220.

8. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TA P, Rempfer C, Bordoli L, et al. SWISS-MODEL: homology modelling of protein structures and complexes, *Nucleic Acids Res* 2018;46:W296-W303.
9. Shang J, Ye G, Shi K, Wan Y, Luo C, Aihara H, Geng Q, Auerbach A, Li F. Structural basis of receptor recognition by SARS-CoV-2, *Nature* 2020;581:221-224.

10. Schrödinger L. The PyMOL Molecular Graphics System, Version 2.5. 2021.

11. Yang TJ, Yu, P.Y., Chang, Y.C., Hsu, S.T.D. Cryo-EM structure of SARS-CoV-2 S-Delta variant (B.1.617.2), one RBD-up conformation 5. 2021.

12. H++. http://biophysics.cs.vt.edu/H++.

13. Anandakrishnan R, Aguilar B, Onufriev AV. H++ 3.0: automating pK prediction and the preparation of biomolecular structures for atomistic molecular modeling and simulations, *Nucleic Acids Res* 2012;40:W537-541.

14. Duan Y, Wu C, Chowdhury S, Lee MC, Xiong G, Zhang W, Yang R, Cieplak P, Luo R, Lee T, et al. A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations, *J Comput Chem* 2003;24:1999-2012.

15. D.A. Case HMA, K. Belfon, I.Y. Ben-Shalom *et al.* AMBER 2018. University of California, San Francisco, 2018.

16. M.J. Abraham TM, R. Schulz, S. Páll, J.C. Smith, B. Hess, E. Lindah. GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers, *SoftwareX* 2015; 1-2:19-25.

17. Lindahl A, Hess, van der Spoel. GROMACS 2020.2 Manual (2020.2). Zenodo. https://doi.org/10.5281/zenodo.3773799.

18. Ermak DL, McCammon JA. Brownian dynamics with hydrodynamic interactions, *The Journal of Chemical Physics* 1978;69:1352-1360.

19. Feller SE, Zhang Y, Pastor RW, Brooks BR. Constant pressure molecular dynamics simulation: The Langevin piston method, *The Journal of Chemical Physics* 1995;103:4613-4621.

20. Martyna GJ, Tobias DJ, Klein ML. Constant pressure molecular dynamics algorithms, *The Journal of Chemical Physics* 1994;101:4177-4189.

21. Ryckaert J-P, Ciccotti G, Berendsen HJC. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes, *Journal of Computational Physics* 1977;23:327-341.
22.Essmann U, Perera L, Berkowitz ML, Darden T, Lee H, Pedersen LG. A smooth particle mesh Ewald method, *The Journal of Chemical Physics* **1995**;**103**:8577-8593.

23.McGibbon RT, Beauchamp KA, Harrigan MP, Klein C, Swails JM, Hernández CX, Schwantes CR, Wang LP, Lane TJ, Pande VS. MDTraj: A Modern Open Library for the Analysis of Molecular Dynamics Trajectories, *Biophys J* **2015**;**109**:1528-1532.

24.Kollman PA, Massova I, Reyes C, Kuhn B, Huo S, Chong L, Lee M, Lee T, Duan Y, Wang W, et al. Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models, *Acc Chem Res* **2000**;**33**:889-897.