TASK Quarterly 28 (3) 2024

Heparin Inclusion in Cyclodextrins: A Combined MD and ITC Study

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`https://doi.org/10.34808/tq2024/28.3/a`

Abstract

Cyclodextrins (CDs) are cyclic oligosaccharides widely used as host molecules capable of forming inclusion complexes with a variety of guest compounds. In this study, for the first time, we investigated the potential interactions between α-, β-, and γ-CD and heparin (HP), a highly sulfated glycosaminoglycan, using a combined theoretical and experimental approach. Understanding that these interactions are relevant because cyclodextrins are widely used as carriers in drug delivery systems and taking into account their limitations toward large charged polysaccharides such as heparin can guide the design of modified CDs for pharmaceutical applications. All-atom molecular dynamics (MD) simulations showed that HP does not enter the CD cavity, instead remaining adsorbed on the external surface at the secondary cavity of the CD. Free energy calculations using LIE and MM-GBSA confirmed that complex formation is enthalpically unfavorable in water, with desolvation penalties outweighing van der Waals and electrostatic contributions. The binding observed in the MD simulations could be, therefore, driven by the entropy. Potential of mean force (PMF) analysis further demonstrated that HP translocation through the γ-CD cavity requires overcoming a high energy barrier of ≈ 23 kcal/mol, indicating that the inclusion is not spontaneous. Complementary isothermal titration calorimetry (ITC) measurements in aqueous buffer also showed negligible enthalpy changes, suggesting that complex formation is not driven by detectable heat effects and is predominantly entropically controlled. Overall, our findings highlight the limitations of natural cyclodextrins in encapsulating large, highly charged polysaccharides such as HP, emphasizing the need for their chemical modifications to enable effective host–guest complexation in such systems potentially designed to encapsulate this cargo.

Keywords:

Cyclodextrin, Heparin, Molecular Dynamics, Isothermal Titration Calorimetry

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1. Introduction

Cyclodextrins (CDs) have a unique ability to form relatively stable inclusion complexes, facilitating the modulation of the physicochemical and biological properties of various low-molecular-weight compounds [1]. The specific linkage of α-(1→4)-D-glucopyranose units gives CDs a hydrophilic exterior surface and a hollow hydrophobic interior, making them suitable as host molecules for a wide range of guest substances. This structural characteristic enables CDs to effectively alter the properties and functions of encapsulated compounds, thus expanding their potential applications in diverse fields [2, 3]. This phenomenon is exploited in various fields, particularly in pharmaceutical applications, where CDs are used to improve the stability, solubility and bioavailability of active substances [4]. CDs are also used in biomedical applications to improve drug delivery and performance [5].

The stability of host–guest inclusion complexes is generally determined by the size of the cyclodextrin cavity, which must effectively accommodate the guest molecule [6]. Hydrophobic interactions have been widely reported to play a key role in stabilizing these complexes [7–9]. These interactions are particularly important for the incorporation of hydrophobic drugs and molecules, as they help to preserve the integrity and effectiveness of the encapsulated compounds.

Although hydrophobic forces usually dominate electrostatic interactions in host–guest complexes, the synergistic effect of both types of intermolecular force can greatly enhance the stability of the complex, particularly when the ligand contains charged groups [10, 11]. We recently demonstrated through two specific cases that the size of the hydrophobic moiety in anions — specifically alkyl sulfates and alkyl sulfonates — critically influences the nature of the intermolecular interactions involved. Our findings suggest that electrostatic interactions predominate in species with shorter hydrophobic tails, whereas van der Waals forces dominate for those with longer chains [12, 13]. In this context, we employed a combined theoretical and experimental approach to investigate potential interactions between cyclodextrins (CDs) and heparin (HP), a highly charged glycosaminoglycan, made up of repetitive disaccharide units of GlcNS(6S)-IdoA(2S). Glycosaminoglycans are located in the extracellular matrix of the cell where interactions with their various protein targets such as cytokines and growth factors participate in key biochemical processes underlying a number of diseases and pathologies such as Alzheimer's, Parkinson's, cancer, tissue regeneration pathologies and the onset of viral infections [14].

This study focuses on analyzing the noncovalent forces involved in potential complex formation between the CD and HP, particularly the role of electrostatic interactions. Furthermore, we have thoroughly examined the contributions of hydrophobic forces and hydrogen-bonding interactions to the stabilization and formation of CD-HP inclusion complexes. The data obtained in this work could be used for the design of CD-based HP transporting molecular systems with their future potential implementation in the development of glycosaminoglycan-based drugs.

2. Materials and Methods

2.1. Molecular Dynamics (MD)

2.1.1 Molecular Dynamics Simulations

All-atom molecular dynamics (MD) simulations were performed with α-, β- and γ-CDs and Heparin dp6 (HP) using the Amber20 software package. The force field GLYCAM06j [15] was used for both CD and HP molecules. The experimental structure of the HP (PDB ID: 1HPN) was used as a template for the construction of the HP fragment. The hexasaccharide was constructed using the xLeap module of AMBER. The HP was randomly placed around the CDs, Na^+ counterions were used to neutralize the system's charge, and an octahedron TIP3P periodic box of 8.0 Å water layer from the box's border to the solute was used to solvate all the complexes. Two energy minimization steps were performed: first, 1500 steepest descents and then 1000 conjugate gradient cycles with harmonic restraints of 100 kcal/mol/ $Å^2$ on the solute, followed by 3000 steepest descents and 3000 conjugate gradient cycles without restraints. Then, the system was heated up to 300K for 10 ps with harmonic restraints of 10 kcal/mol/ $Å^2$ on solute and equilibrated for 100 ps at 300 K and 10^5 Pa in an isothermal, isobaric ensemble (NTP). After this, MD simulations were carried out in the same NTP ensemble for 150 ns for the system with the SHAKE algorithm for all the covalent bonds containing hydrogen atoms, and the Particle Mesh Ewald method was used to treat electrostatics. The trajectories were analyzed by the cpptraj module of AMBER tools.

2.1.2 Binding Free Energy Calculations

Binding free energy calculations were performed for all systems with the obtained MD trajectories using Linear Interaction Energy (LIE) [16]. The dielectric constant was set at 80, and the default weights were used with the cpptraj scripts from Amber20. The Molecular Mechanics Generalized Born Surface Area (MM-GBSA) free energy calculations were performed using a model with the surface area and Born radii as default parameters in the igb=2 model in Amber20 [17]. All frames from MD production runs were analyzed.

2.1.3 Umbrella Sampling and the Potential of Mean Force

The Umbrella Sampling (US) approach was applied to compute the free energy along the dissociation pathway for γ-CD, the only CD whose cavity size allowed the HP chain to be placed into it. The distance between the center of mass of the CD backbone and one of terminal sulfur atoms of the HP was calculated and used as the reaction coordinate. The initial distance was increased by 1 Å for 40 windows, and the constant forces were set to 8 kcal/mol/ Å^2. Conformational sampling in these windows was achieved by applying an external biasing potential. The increase of the reaction coordinate corresponds to the process of guiding the HP through the cavity of the γ-CD, allowing the free energy profile of this translocation to be estimated. Once the US simulations were finished, the data collected from the separate simulations windows were used to calculate the potential of mean force (PMF) profile for the molecule transition process using the weighted histogram analysis method (WHAM) [18]. For each window, the MD simulation was carried out for 10 ns. For the analysis with WHAM, the tolerance iteration was 0.001 and the temperature was 300 K.

2.1.4 Data Analysis and Visualization

Data analysis was performed with R-package [19], and the visualization was performed using VMD [20].

2.2. Reagents

β-CD (≥97%, CAS: 7585-39-9) was purchased from Sigma Aldrich (Poland), γ-CD (≥98%, CAS: 17465-86-0) was purchased from Apollo Scientific (UK). The reagents were used without further purification. The HP oligosaccharides were obtained from Iduron (Manchester, UK). All the reagents were prepared freshly before each measurement in a 10 mM phosphate buffer (pH 7.0, no added salt).

2.3. Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) experiments were performed at a temperature of 298.15 K using an Auto-ITC instrument (MicroCal, GE Healthcare, Northampton, USA). The sample cell contained a HP solution at a concentration of 0.2 mM. The titrations involved the addition of 4 mM solutions of either β-CD or γ-CD as the titrant. Each injection was conducted over 20 seconds, with 240-second pauses between injections to allow for equilibrium. The heat of dilution for the CDs was measured separately in control experiments and subsequently subtracted from the titration data.

3. Results

3.1. Molecular Dynamics

The interaction of CDs (α, β, and γ-CD) with HP was analyzed by the MD approach, and across all 1:1 complexes tested, no evidence of HP inclusion inside the cyclodextrin cavities was observed during the 150 ns. Instead, HP consistently remained bound at the outer surface of the CDs (Fig. 1), suggesting that its strong electrostatic character prevents spontaneous encapsulation. The binding occurs at the secondary cavity of the CD, which is to be expected due to the higher positive potential near this cavity [12], which is caused by the availability of two hydroxyl groups from each glucose residue. In turn, only one such hydroxyl group (in the C6 position) is available in the primary cavity.

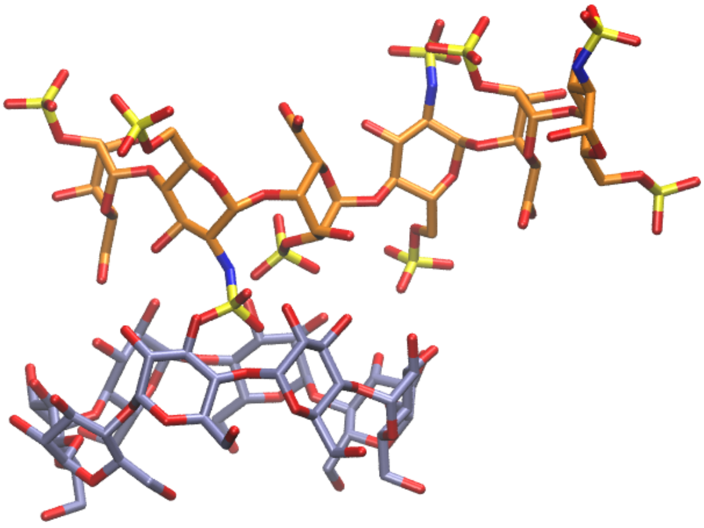

Figure 1: A representative structure of γ-CD (carbons in ice blue) complexed with the HP (carbons in orange) obtained in the MD simulation.

Free energy analyses using both LIE and MM-GBSA (Table 1) support this conclusion. Both α- and β-CD showed nearly neutral ΔG values (1.4 ± 3.7 and 2.6 ± 4.6 kcal/mol, respectively) upon binding the HP, consistent with weak and transient associations. γ-CD displayed strongly favorable van der Waals (-23.4 ± 16.3 kcal/mol) and electrostatic contributions (-82.7 ± 54.7 kcal/mol). However, these favorable interactions were offset by the large desolvation penalty of HP (113.4 ± 70.1 kcal/mol), leading to an overall unfavorable ΔG (7.2 ± 6.8 kcal/mol). These findings indicate that the strong hydration of HP sulfate groups is the primary thermodynamic barrier to inclusion. The fact that the association was observed in the MD simulations but the calculated LIE and MM-GBSA values are not favorable is due to the entropic component of binding, which is significantly neglected by these free energy calculation approaches.

Table 1: MM-GBSA binding free energies for CD-HP complexes (kcal/mol).

CD	ΔG_{LIE}	$\Delta G_{\mathrm{MM\text{-}GBSA}}$	ΔG_{VDW}	ΔG_{EEL}	ΔG_{GAS}	ΔG_{SOLV}
α	10.9 ± 7.6	1.4 ± 3.7	-5.3 ± 6.9	-21.8 ± 29.8	-27.2 ± 35.2	28.6 ± 35.7
β	13.4 ± 8.2	2.6 ± 4.6	-6.7 ± 10.3	-27.8 ± 43.6	-34.4 ± 52.9	37.0 ± 55.9
γ	13.1 ± 8.2	7.2 ± 6.8	-23.4 ± 16.3	-82.7 ± 54.7	-106.2 ± 67.5	113.4 ± 70.1

These results reinforce the idea that although γ-CD provides a larger and more accommodating interaction surface, the high polarity and strong hydration of HP impose a significant energetic cost that prevents stable complex formation. These results are consistent with the visualization of MD trajectories, where HP was observed to predominantly interact at the CD surface without spontaneous penetration into the cavity.

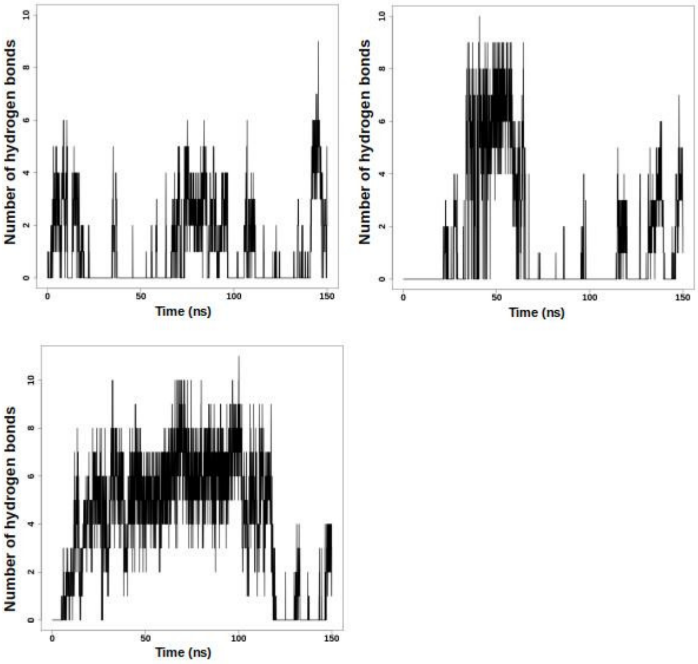

Figure 2: Number of hydrogen bonds between (a) α-CD (b) β-CD and (c) γ-CD and HP.

Hydrogen bond analysis provided additional details on the interaction patterns in the analyzed systems (Fig. 2). For α-CD, hydrogen bonding was weak and transient, typically fluctuating between 2 and 4, and only rarely exceeding 6 bonds. β-CD showed stronger surface interactions, with frequent hydrogen bond counts up to 10, particularly during the first half of the simulation. However, these interactions also decayed over time of the simulation suggesting that there are association/dissociation events occurring within this time scale. By contrast, γ-CD displayed the most persistent hydrogen bonding, often ranging between 6 and 10 throughout much of the trajectory, though this interaction also weakened toward the end. These results suggest that the lower curvatures of the γ-CD external surface allow for closer and more stable surface contacts with HP through H-bonding, even though the inclusion within the cavity was not observed.

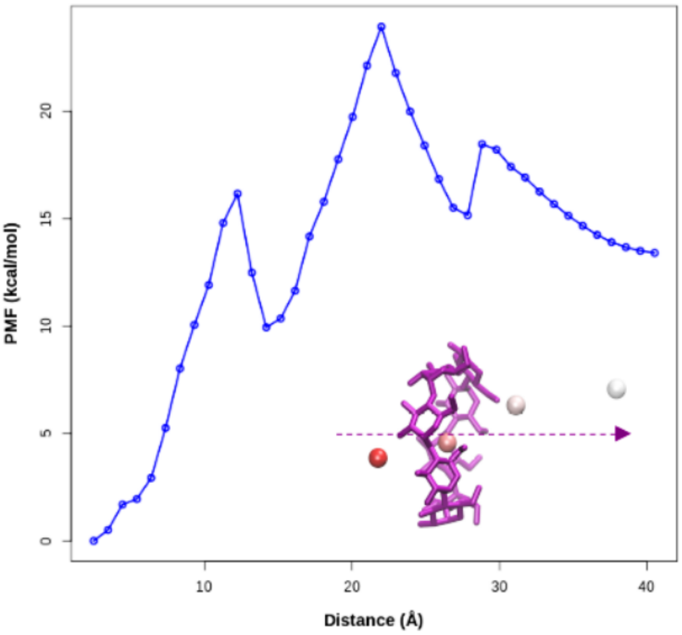

Figure 3: PMF profile of energy required to pass HP through γ-CD ring (blue). The inset represents the γ-CD ring (purple) and the HP is represented by its C1 of the reducing end, and the pathway is displayed from an initial distance of 2 Å (red, starting frame) to a distance of 28 Å (white, third energy peak)..

The potential of mean force (PMF) analysis obtained from Umbrella Sampling simulations supports this finding (Fig. 3). While the HP reducing end was forced to pass through the γ-CD cavity, translocation required overcoming an energy barrier of approximately 23 kcal/mol. The PMF profile revealed three distinct barriers: the first (≈ 12 kcal/mol) corresponded to HP retaining the partial occupancy in the cavity interior, the second (≈ 23 kcal/mol) to HP at the cavity border, and the third (≈ 20 kcal/mol) to HP leaving the cavity back into the solvent. These results confirm that inclusion of HP within CD cavity is energetically costly and unlikely under spontaneous conditions, with surface interactions representing the dominant binding mode.

3.2. Isothermal Titration Calorimetry

The investigation of systems involving CDs is often a complex and multifaceted task. Although numerous conventional analytical methods are available for studying CD-guest interactions in aqueous solutions, the selection of the most appropriate technique depends on the specific characteristics of the guest molecule [21]. Among the various analytical approaches, ITC stands out as one of the most powerful tools for directly determining key thermodynamic parameters of inclusion complexes, such as stoichiometry, binding constant, and binding enthalpy [22–24]. Representative binding isotherms for the interaction of the investigated CDs with HP in a 10 mM phosphate buffer solution at pH 7.0 are shown in Fig. 4. It has been observed that the heat effects associated with the mixture of CDs and HP are very small and exothermic across the entire range of the experimental CD:HP molar ratios. Moreover, the magnitude of these energetic effects is comparable to the heat changes resulting from the dilution of the titrant. This suggests that the enthalpy change (ΔH) associated with complex formation is approximately zero ($\Delta H \approx 0$), which is in agreement with the data obtained from the MD simulations. In such a scenario, the formation of the complexes is primarily driven by entropy changes ($T\Delta S$), with a positive entropy contribution sufficiently large to offset the negligible enthalpy variations. Consequently, under these conditions, the calorimetry data do not provide reliable information about the nature or strength of the interactions between CD and HP, limiting the usefulness of calorimetry for characterizing these particular systems.

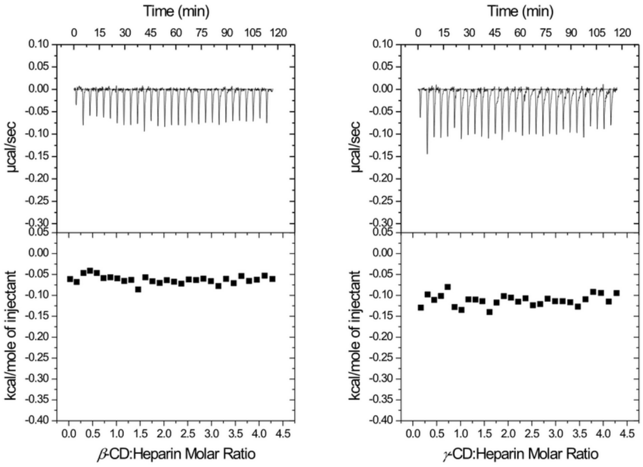

Figure 4: Calorimetric titration isotherms of binding interactions of β-CD and γ-CD with HP in a 10 mM phosphate buffer (pH 7.0, no added salt), at 298.15 K.

These analyses demonstrate that natural cyclodextrins have a very limited capacity to encapsulate HP. γ-CD shows the closest approach to a meaningful interaction, but the substantial energetic cost associated with desolvating the highly sulfated HP chain ultimately prevents efficient complexation.

4. Summary

To conclude, in this work we combined MD and ITC approaches to analyze the potential binding between CDs and HP. The data obtained with both methods indicate rather weak entropy-driven interactions, suggesting that the binding does not occur through the formation of an inclusion complex. Instead, the HP molecule binds predominantly to the secondary cavity of the CD via unspecific hydrogen bonds. Our results underscore the inherent limitations of natural CDs in encapsulating large highly charged polysaccharides like HP, and point to the necessity of their chemical modification to achieve efficient host–guest complexation in systems aimed at delivering such macromolecular cargo.

Despite the limitations of the encapsulation of HP, our findings provide valuable understanding of the physicochemical boundaries of host-guest complexation involving highly charged molecules. While most CD studies focus on small hydrophobic guests, our results extend this knowledge to explore how electrostatic repulsion and solvation effects limit inclusion for large glycosaminoglycans, which can be used in the future design of derivatized CDs.

Acknowledgements

This research was funded by the National Science Centre of Poland (grant number UMO-2023/49/B/ST4/00041) and Mayor of Gdańsk Fahrenheit Scholarship for International PhD Students. We would like to thank professor Daniel Huster (Leipzig University) for providing the heparin oligosaccharides.

References

[1] Álvaro Sarabia-Vallejo, M. del Mar Caja, A. I. Olives, M. A. Martín, and J. C. Menéndez, "Cyclodextrin inclusion complexes for improved drug bioavailability and activity: Synthetic and analytical aspects," *Pharmaceutics*, vol. 15, p. 2345, 9 2023.

[2] A. Cid-Samamed, J. Rakmai, J. C. Mejuto, J. Simal-Gandara, and G. Astray, "Cyclodextrins inclusion complex: Preparation methods, analytical techniques and food industry applications," *Food Chemistry*, vol. 384, p. 132467, 8 2022.

[3] A. M. Musuc, "Cyclodextrins: Advances in chemistry, toxicology, and multifaceted applications," *Molecules*, vol. 29, p. 5319, 11 2024.

[4] G. Kali, S. Haddadzadegan, and A. Bernkop-Schnürch, "Cyclodextrins and derivatives in drug delivery: New developments, relevant clinical trials, and advanced products," *Carbohydrate Polymers*, vol. 324, p. 121500, 1 2024.

[5] M. A. Esteso and C. M. Romero, "Cyclodextrins: Properties and applications," *International Journal of Molecular Sciences*, vol. 25, p. 4547, 4 2024.

[6] A. Buvári-Barcza, “Influence of the guests, the type and degree of substitution on inclusion complex formation of substituted -cyclodextrins,” *Talanta*, vol. 49, pp. 577–585, 7 1999.

[7] E. Junquera, D. Ruiz, and E. Aicart, “Role of hydrophobic effect on the noncovalent interactions between salicylic acid and a series of -cyclodextrins,” *Journal of Colloid and Interface Science*, vol. 216, pp. 154–160, 8 1999.

[8] O. E. Nicolaescu, I. Belu, A. G. Mocanu, V. C. Manda, G. Rău, A. S. Pîrvu, C. Ionescu, F. Ciulu-Costinescu, M. Popescu, and M. V. Ciocîlteu, “Cyclodextrins: Enhancing drug delivery, solubility and bioavailability for modern therapeutics.,” *Pharmaceutics*, vol. 17, 2 2025.

[9] V. Aiassa, C. Garnero, A. Zoppi, and M. R. Longhi, “Cyclodextrins and their derivatives as drug stability modifiers,” *Pharmaceuticals*, vol. 16, p. 1074, 7 2023.

[10] H. Schneider and I. Theis, “Additivities of electrostatic and hydrophobic interactions in host–guest complexes,” *Angewandte Chemie International Edition in English*, vol. 28, pp. 753–754, 6 1989.

[11] R. Paul and S. Paul, “Synergistic host–guest hydrophobic and hydrogen bonding interactions in the complexation between endo-functionalized molecular tube and strongly hydrophilic guest molecules in aqueous solution,” *Physical Chemistry Chemical Physics*, vol. 20, pp. 16540–16550, 2018.

[12] M. M. Kogut, O. Grabowska, D. Wyrzykowski, and S. A. Samsonov, “Affinity and putative entrance mechanisms of alkyl sulfates into the -cd cavity,” *Journal of Molecular Liquids*, vol. 364, p. 119978, 10 2022.

[13] O. Grabowska, M. M. Kogut-Günthel, S. A. Samsonov, D. Wyrzykowski, and J. Makowska, “-cyclodextrin saves lysozyme from the clutches of 1-alkylsulfonates,” *Journal of Molecular Liquids*, vol. 428, p. 127543, 6 2025.

[14] S. Perez, O. Makshakova, J. Angulo, E. Bedini, A. Bisio, J. L. de Paz, E. Fadda, M. Guerrini, M. Hricovini, M. Hricovini, F. Lisacek, P. M. Nieto, K. Pagel, G. Paiardi, R. Richter, S. A. Samsonov, R. R. Vivès, D. Nikitovic, and S. R. Blum, “Glycosaminoglycans: What remains to be deciphered?,” *JACS Au*, vol. 3, pp. 628–656, 3 2023.

[15] K. N. Kirschner, A. B. Yongye, S. M. Tschampel, J. González-Outeiriño, C. R. Daniels, B. L. Foley, and R. J. Woods, “Glycam06: A generalizable biomolecular force field. carbohydrates,” *Journal of Computational Chemistry*, vol. 29, pp. 622–655, 3 2008.

[16] T. Hansson, J. Marelius, and J. Åqvist, “Ligand binding affinity prediction by linear interaction energy methods,” *Journal of Computer-Aided Molecular Design*, vol. 12, pp. 27–35, 1 1998.

[17] V. Tsui and D. A. Case, “Theory and applications of the generalized born solvation model in macromolecular simulations,” *Biopolymers*, vol. 56, pp. 275–291, 2000.

[18] S. Kumar, J. M. Rosenberg, D. Bouzida, R. H. Swendsen, and P. A. Kollman, “The weighted histogram analysis method for free-energy calculations on biomolecules. i. the method,” *Journal of Computational Chemistry*, vol. 13, pp. 1011–1021, 10 1992.

[19] R. C. Team *et al.*, “R: A language and environment for statistical computing,” *R foundation for statistical computing, Vienna, Austria*, 2021.

[20] W. Humphrey, A. Dalke, and K. Schulten, “Vmd: Visual molecular dynamics,” *Journal of Molecular Graphics*, vol. 14, pp. 33–38, 2 1996.

[21] P. Mura, “Analytical techniques for characterization of cyclodextrin complexes in aqueous solution: A review,” *Journal of Pharmaceutical and Biomedical Analysis*, vol. 101, pp. 238–250, 12 2014.

[22] E. Bertaut and D. Landy, “Improving itc studies of cyclodextrin inclusion compounds by global analysis of conventional and non-conventional experiments,” *Beilstein Journal of Organic Chemistry*, vol. 10, pp. 2630–2641, 11 2014.

[23] P. Brocos, X. Banquy, N. Díaz-Vergara, S. Pérez-Casas, Ángel Piñeiro, and M. Costas, “A critical approach to the thermodynamic characterization of inclusion complexes: Multiple-temperature isothermal titration calorimetric studies of native cyclodextrins with sodium dodecyl sulfate,” *The Journal of Physical Chemistry B*, vol. 115, pp. 14381–14396, 12 2011.

[24] K. Bouchemal and S. Mazzaferro, “How to conduct and interpret itc experiments accurately for cyclodextrin–guest interactions,” *Drug Discovery Today*, vol. 17, pp. 623–629, 6 2012.