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SAMPL9 blind predictions using nonequilibrium alchemical approaches

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We present our blind predictions for the Statistical Assessment of the Modeling of Proteins and Ligands (SAMPL), 9th challenge, focusing on binding of WP6 (carboxy-pillar[6]arene) with ammonium/diammonium cationic guests. Host-guest binding free energies have been calculated using the recently developed virtual double system single box approach, based on the enhanced sampling of the bound and unbound end-states followed by fast switching nonequilibrium alchemical simulations [M Macchiagodena, M Pagliai, M Karrenbrock, G Guarnieri, F Iannone, Procacci, *J Chem Theory Comput*, 16, 7260, 2020]. As far as Pearson and Kendall coefficients are concerned, performances were acceptable and, in general, better than those we submitted for calixarenes, cucurbituril-like open cavity, and beta-cyclodextrines in previous SAMPL host-guest challenges, confirming the reliability of nonequilibrium approaches for absolute binding free energy calculations. In comparison with previous submissions, we found nonetheless a rather large mean signed error that we attribute to the way the finite charge correction was addressed through the assumption of a neutralizing background plasma.

Keywords: SAMPL9, binding free energy, Non-equilibrium, Crooks theorem, fast switching, Hamiltonian Replica Exchange, HREX, Solute Tempering, WP6

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I. INTRODUCTION

The Statistical Assessment of the Modeling of Proteins and Ligands (SAMPL) is a set of community-wide blind challenges aimed at advancing the computational techniques in drug design.¹⁻⁵ New experimental data, such as dissociation free energies, hydration free energies, acid-base dissociation constants, or partition coefficients are withheld from participants until the prediction submission deadline, so that the true predictive power of methods can be assessed.

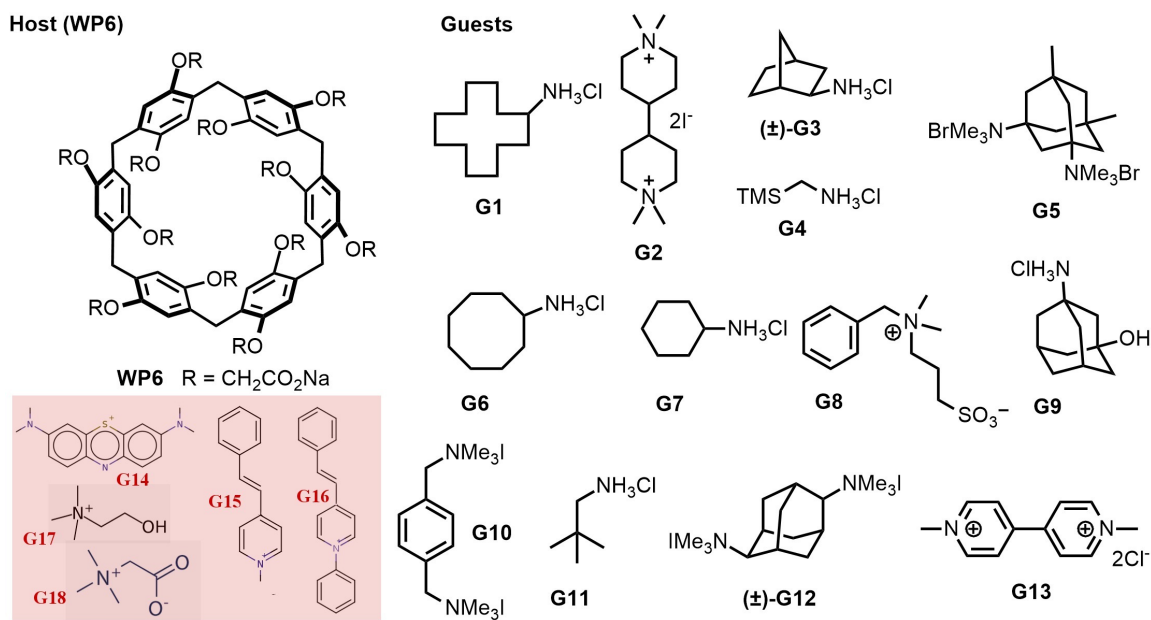


FIG. 1. Host-guest SAMPL9 challenge. In the shaded red area on the bottom left corner, the molecules with known binding affinities used for pre-assessment are reported (see text).

In the latest 9th challenge, participants were required to predict the binding affinities of thirteen ammonium/diammonium cationic ligands (guests) vs WP6, a water-soluble toroidal macrocyclic host molecule (see Figure 1). Host-guest experimental binding affinities, measured by Isothermal Titration Calorimetry (ICT) at pH=7.4 were disclosed by November 2021 and are now reported in Ref.⁶. Actually, the binding affinity of G13 (Paraquat) was available on literature at physiological pH^{7,8} before the submission deadline. The host WP6, never featured in previous SAMPL challenges, is structurally similar to cucurbit[n]urils (CBn) used in SAMPL6³ and SAMPL8⁹ with important differences related to the electrostatic interactions. Unlike the neutral CBn host, the quasi-D6h WP6 bears six carboxylated moieties on the upper and lower rims that can be in part protonated at pH=7.4.^{7,8} As the pKa's of WP6 were not available, such a feature represented an

important challenge in the blind prediction of WP6-guest binding affinities. Participants were in fact requested to deal with this complication.

In this report, we present the dissociation free energies of the fully anionic WP6⁻¹² for the thirteen cationic guests of Figure 1 plus five additional guest molecules with known dissociation constants, namely methylene blue (G14)¹⁰, GNF-Pf-3194 (G15)¹¹, M2 (G16)¹¹, choline (G17)¹² and betaine (G18)¹². The calculations have been performed on the CRESCO6-ENEA cluster¹³ in Portici (Italy) using the so-called virtual Double System Single Box (vDSSB) method¹⁴⁻¹⁷, based on a production of a swarm of concurrent nonequilibrium (NE) alchemical simulations that are started from end-state canonical configurations sampled using Hamiltonian Replica Exchange (HREM)¹⁸.

II. METHODS

A. Estimation of the protonation state of WP6

As stated in the introduction, the twelve pKa’s of WP6 host were not known before the SAMPL9 deadline for submission. In presence of multiple host-guest complexes with various protonation states, the overall observed association constant for Gn-WP6 complexes is given by

$$K_a = \sum_k W_k K_a^{(k)} \tag{1}$$

where $K_a^{(k)}$ is the association constant for the k -th protonated WP6 species and W_k is the corresponding normalized weight. The Gn-WP6 dissociation free energy should be hence calculated as

$$\Delta G_d = RT \ln \sum_k W_k e^{\beta \Delta G_d^{(k)}} \tag{2}$$

where $\Delta G_d^{(k)}$ is the dissociation free energy with the host in the k -th protonation state. The predicted pKa of the WP6 template monobasic acid 2-(2,5-dimethylphenoxy) acetic acid is 3.23¹⁹. We assumed a pKa distribution modulated by the so-called statistical factor²⁰ for twelve equivalent protonation sites

$$\text{pKa}_n = \text{pKa}_1 - \log(n/13 - n) \tag{3}$$

where $\text{pKa}_1 = 3.23$ is that of the monobasic acid. We obtained that the prevalent species at pH=7.4 is the WP6⁻¹² anion with all deprotonated carboxylated groups with a weight $W_{-12} = 0.98$, in

surprisingly good agreement, despite the crudeness of our approach, with the experimental value of $W_{-12} = 0.95$ released after the submission deadline.²¹

We hence computed the dissociation constant for the $WP6^{-12}$ species only, related to the chemical equilibrium



It should be nonetheless stressed that the large weight of the fully deprotonated species, $W=0.95$, does not guarantee that the experimental dissociation free energy corresponds to the dissociation free energy of the equilibrium Eq. 4. If the dissociation free energy of any of the other simultaneous chemical equilibria, $WP6^{-n}Gn \leftrightarrow WP6^{-n} + Gn$ with $n = 1 \dots 11$, is much *larger* than that corresponding to the equilibrium Eq. 4, then, according to Eq. 2, the overall experimental free energy can be significantly higher than that matching the equilibrium Eq. 4. So our dissociation free energy, computed for the equilibrium 4, should be viewed as a *lower bound* for the true experimental dissociation free energy.

B. vDSSB approach for absolute dissociation free energies

The vDSSB methodology is thoroughly described in Ref.^{15,16}. In brief, the method consists of two massively parallel computational steps, the HREM stage and the nonequilibrium alchemical stage. The HREM stage relies on the enhanced sampling of the host-guest bound state (with the ligand at full coupling) in explicit water and of the isolated guest molecule. The initial configurations for the unbound state are prepared by combining the HREM-sampled gas-phase (decoupled) ligand snapshots with a pre-equilibrated box filled with explicit water. HREM uses $n = 16$ and $n = 8$ replicas for the bound and unbound states respectively, with the lowest scaling factor of $S = 0.1$ (corresponding to the highest temperature of 3000 K) involving only the intra-solute bonded and non-bonded interactions²². The scaling factors along the replica progression are computed according to the protocol $S_m = S^{(m-1)/n}$ with $m = 1 \dots n$. Only the scaling factors are exchanged among neighboring replicas to minimize the communication overhead on the MPI layer. The HREM simulations are run for 48 ns and 16 ns for the bound and gas-phase state, respectively with exchange rates in the range 25%-50%.

In the NE alchemical stage, the bound state leg of the alchemical cycle is performed by rapidly decoupling the bound ligand in a swarm of 360 independent NE simulations, each lasting 1.44 ns. The unbound leg of the cycle is done by growing (recoupling) the ghost ligand in the solvent in

480 NE alchemical simulations lasting 0.36 ns. Electrostatic and Lennard-Jones interactions are switched off/on sequentially, in order to separate the corresponding contributions to the binding. For further details on the ligand coupling/decoupling protocols, we refer to Ref.^{15,16}.

The final bound and unbound alchemical work distributions are combined in the convolution

$$P(W) = P_b * P_u(W) = \int dw P_b(W) P_u(W - w) dw \quad (5)$$

as if the two independent processes of the ligand annihilation in the bound state and of the ligand growth in the pure solvent occurred in the same box (hence the name vDSSB), with one ligand on the host and the other in the far distant bulk.

The dissociation free estimate can be performed by way of the Jarzynski identity²³

$$\Delta G_{\text{vDSSB}} = -RT \ln \int P(W) e^{-\beta W} dW \quad (6)$$

or using the Crooks theorem²⁴ in the assumption that the convolution can be described by a mixture of normal distributions^{15,25}, i.e

$$\Delta G_{\text{vDSSB}} = -RT \ln \sum_i c_i e^{-\beta(\mu_i - \frac{1}{2}\beta\sigma_i^2)} \quad (7)$$

where c_i , μ_i , σ_i^2 are the normalized weight, mean and variance of the i -th normal component, determined via the expectation-maximization algorithm^{26,27}. Estimates based on eq. 6 or eq. 7 are used depending on the width and character of the work distributions as assessed by the Anderson-Darling normality test²⁸. The free energy estimates are corrected for a volume²⁹⁻³¹ and a charge term^{30,32} (for non-neutral ligand) given by

$$\Delta G_{\text{vol}} = RT \ln \left[4\pi \frac{(2\sigma)^3}{3V_0} \right] \quad (8)$$

$$\Delta G_{\text{fs}} = -\frac{\pi}{2\alpha^2} \left\{ \frac{[Q_H^2 - (Q_H + Q_G)^2]}{V_{\text{BOX}}^{(b)}} + \frac{Q_G^2}{V_{\text{BOX}}^{(u)}} \right\} \quad (9)$$

where σ is the standard deviation of the host-guest COM-COM distance distribution in the bound state, V_0 is the standard state volume, Q_G , Q_H are the net charges of the guest and the host, $V_{\text{BOX}}^{(b)}$, $V_{\text{BOX}}^{(u)}$ and are the mean volume of the MD box for the bound and unbound state respectively, and α is the Ewald convergence parameter. The effectiveness of the correction Eq. 9 for finite size effects when dealing with charged ligands has been amply assessed in Ref.³⁰, while the origin of the binding volume term Eq. 8 has been discussed in Refs.^{29,31,33}. The final blind prediction for the standard dissociation free energy is given by

$$\Delta G_d^0 = \Delta G_{\text{vDSSB}} + \Delta G_{\text{vol}} + \Delta G_{\text{fs}} \quad (10)$$

C. Simulation setup and parameters

Guests and host PDB files were taken from the SAMPL9 GitHub site. For the two chiral compounds, the G3 guest (2-bicyclo[2.2.1]heptanyl]azanium) corresponds to the 1R 2R 4S diastereoisomer. The G12 guest (Hexamethyladamantane-2,6-diammonium) has the 2 and 6 carbon atoms of the R type. The Force Field (FF) parameters and topology of the host and guests molecules were prepared using the PrimaDORAC interface³⁴ based on the GAFF2³⁵ parameter set. For G4, the silicon-related parameters were taken from Ref.³⁶. The initial bound state was prepared using the Autodock Vina code³⁷. The bound complexes and the ghost ligands were solvated in about 1600 and 512 OPC3³⁸ water molecules, respectively. Long-range electrostatic interactions were treated using the Smooth Particle Mesh Ewald method (SPME)³⁹. A background neutralizing plasma was assumed within the SPME method³². The cut-off of the Lennard-Jones interactions was set to 13 Å. All simulations, HREM or nonequilibrium, were performed in the NPT ensemble in standard conditions using an isotropic Parrinello-Rahman Lagrangian⁴⁰ and a series of Nosé thermostats⁴¹ for pressure and temperature control, respectively. Bonds constraints were imposed on X-H bonds only, where X is a heavy atom. All other bonds were assumed to be flexible. In the bound state simulations (HREM or NE) the guest and the host center of mass (COM) were tethered by a weak harmonic potential with a force constant of $K \simeq 0.06$ kcal/mol Å⁻², corresponding to a ligand allowance volume of $\simeq 600$ Å³. All simulations have been done using the hybrid OpenMP-MPI program ORAC⁴² on the CRESCO6 cluster¹³. ORAC Input files for the HREM simulations are provided on the Zenodo platform (<https://zenodo.org/record/5891191>).

III. RESULTS

In Figure 2 we show the host-guest COM-COM distance distributions in the bound state for all thirteen SAMPL9 pairs as obtained from the target state HREM sampling of 48 ns. According to Eq. 8, the binding site volume is calculated from the variance of the COM-COM distribution as $V_{\text{site}} = 4\pi \frac{(2\sigma)^3}{3}$. As it can be seen, despite the weak tethering COM-COM harmonic potential and the correspondingly large allowance radius of $\simeq 8$ Å (exceeding substantially the radius of the WP6 cavity of $\simeq 5.4$ Å), the guests never leave the WP6 pocket, with a standard deviation of the COM-COM distribution ranging from $\simeq 0.2$ Å (G7) to $\simeq 0.9$ Å (G10). As discussed in Ref.³¹, the HREM approach with *intrasolute* (ligand and host) scaling does not accelerate passive diffusion

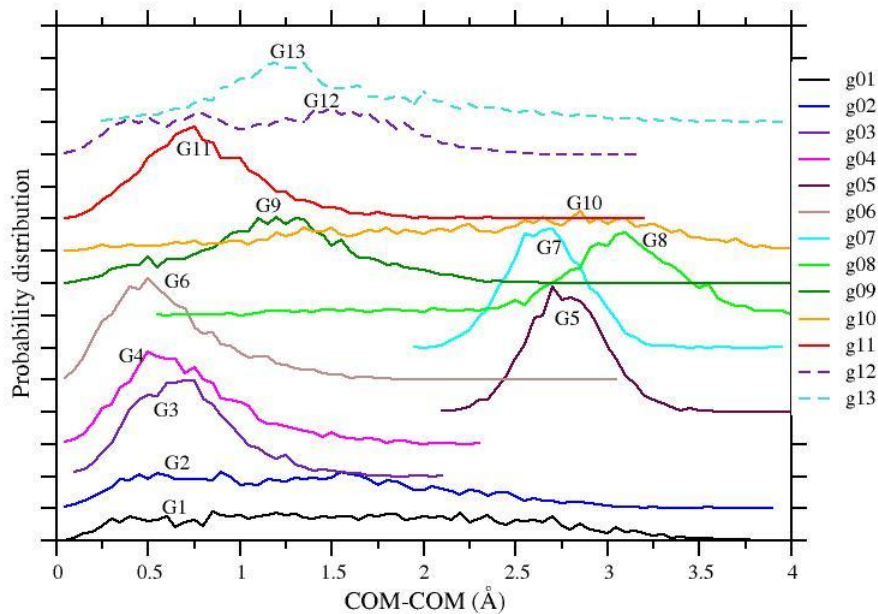


FIG. 2. Host-guest COM-COM distance distributions obtained from the HREM sampling of the bound state.

with little impact on the k_{off} and on the ligand residence time (in the range of the microsecond to milliseconds for all ligands). On the Zenodo platform (<https://zenodo.org/record/5891191>) we provide the HREM-generated trajectories of the bound state for all thirteen guest-host systems.

In Table I we have collected all the quantities that have been used in the determination of the estimate of the standard dissociation free energy, Eq. 10, namely the charge and volume corrections (Eq. 8 and Eq. 9), the mean and variance of the bound and unbound work distributions as well as their character as assessed by the Anderson-Darling test. The bound and unbound work distributions, along with their convolution Eq. 5, are reported for all guests of Table I in the Electronic Supporting Information (ESI). The corresponding raw data of the work can be found on the Zenodo site (<https://zenodo.org/record/5891191>). The last column of Table I indicates the type of estimate for the alchemical dissociation free energies ΔG_{VDSSB} of the ranked submission: G_b refers to a Gaussian estimate, S_3 is an estimate based on the Gaussian mixture, Eq. 7, with three components, and finally the estimate $S_J + B$ is based on the Jarzynsky exponential average, Eq. 6.

guest	ΔG_{vol}	ΔG_{fs}	$\langle W_b \rangle$	σ_b	$\langle W_u \rangle$	σ_u	AD_b	AD_u	σ_{BU}	Est. type
G1	-2.6	-2.0	75.9	3.2	-53.0	1.9	2.32	2.96	3.73	$S_J + B$
G2	-2.9	-4.3	147.6	2.3	-121.2	1.1	0.41	0.24	2.53	S_3
G3	-4.5	-2.0	78.5	2.8	-56.8	0.8	2.57	0.63	2.88	S_3
G4	-4.2	-2.0	73.8	2.2	-56.8	0.8	0.38	0.31	2.35	G_b
G5	-4.5	-4.3	144.0	2.3	-122.6	1.3	0.50	0.29	2.61	S_3
G6	-4.3	-2.0	78.6	2.9	-55.4	0.9	1.34	0.45	3.02	$S_J + B$
G7	-5.0	-2.0	82.6	3.0	-57.0	0.8	0.46	0.90	3.14	S_3
G8	-3.3	0.0	75.5	3.2	-49.9	1.6	1.34	0.50	3.57	S_3
G9	-3.6	-2.0	83.1	2.8	-59.4	1.0	0.67	0.47	2.99	$S_J + B$
G10	-2.6	-4.3	143.7	2.3	-119.5	1.1	0.65	0.23	2.51	S_3
G11	-4.2	-2.0	76.3	2.3	-58.5	0.8	0.81	0.70	2.41	S_3
G12	-3.4	-4.3	151.3	2.1	-122.1	1.1	0.25	0.22	2.37	G_b
G13	-3.0	-4.3	144.5	2.4	-125.4	0.9	0.28	0.18	2.60	G_b
G14	-2.8	-2.0	44.5	1.9	-21.2	1.1	0.30	0.31	2.18	G_b
G15	-2.8	-2.0	51.7	2.1	-32.1	0.9	0.42	0.29	2.31	S_3
G16	-2.7	-2.0	49.1	2.0	-27.5	1.1	0.73	0.40	2.26	S_3
G17	-4.3	-2.0	57.3	1.6	-40.9	0.7	0.45	0.36	1.72	S_3
G18	-3.7	0.0	44.5	1.7	-33.4	0.9	1.22	0.52	1.91	$S_J + B$

TABLE I. Salient data for vDSSB predictions. ΔG_{vol} , standard state correction; ΔG_{fs} , finite size correction for charged guest molecule; $\langle W_{b/u} \rangle$, $\sigma_{b/u}$, AD_b , mean NE work, standard deviation and Anderson Darling test for the bound and unbound states; σ_{BU} , standard deviation of the convolution; Est. type, estimate type (see text). Energy unit is in kcal/mol.

Ideally, such estimates should all converge to the same value when $n \rightarrow \infty$ and/or the duration of the NE process $\tau \rightarrow \infty$. For finite samples, their precision and accuracy are strongly affected by the spread (connected to the dissipation of the process³¹) and the character of the work distributions. The criteria for selecting the estimate type for each pair were the following: i) if both the bound and unbound work distributions prior to the convolution pass the Anderson-Darling test with a p-value⁴³ greater than 50% ($A^2 < 0.3$), then the Gaussian estimate is adopted; otherwise, ii) if the uncertainty is less than 2 kcal/mol we use S_3 ; otherwise iii) we use the Jarzynski estimate

$S_J + B$, where to Eq. 6 is added a correction due to the bias, recalling that the *positive bias* bias in exponential averaging of the NE work is inescapable^{44,45}. The convolution, by boosting the

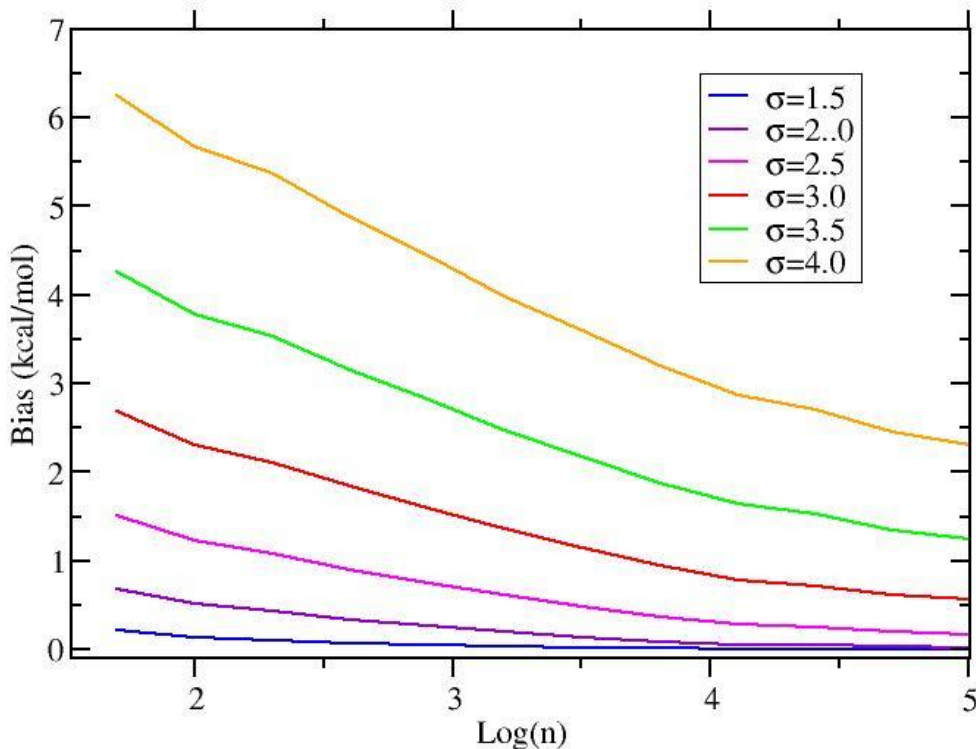


FIG. 3. Bias of the exponential average as a function of n and σ

sample size to $n = n_b \times n_u$, tames the bias but does not suppress it, notably when the width of the resulting distribution is large. As one can see from Table I and Figures S1-S18 of the ESI, in general, the width of the convolution is somewhat larger than the width of the *bound state* distribution, since the variance of the unbound-state work distribution, σ_u^2 , is much smaller than that of bound-state work distribution, σ_b^2 , even if the duration time of the ligand annihilation in the bound-state process (1.44 ns) is much longer than that of the ligand growth process in the unbound-state (0.36 ns). As the NE growth work for the unbound-state and the NE annihilation work for the bound-state are two *independent* random variables, the variance of the convolution is given by $\sigma_{BU} = \sigma_b^2 + \sigma_u^2$. From the variance of the convolution σ_{BU}^2 , the bias can be readily estimated by a generating a sample of *normal distributions* with zero mean and variance σ_{BU}^2 and

by computing the bias as

$$B(\sigma, n) = \left\langle \frac{1}{n} \sum^n e^{-\beta W} \right\rangle + \frac{1}{2} \sigma_{bu}^2 \quad (11)$$

where the exponential sum is averaged over all generated normal distributions. In Figure 3, we report the bias, Eq. 11 as a function of the number of points and of the variance. In our case, $n = 360 \times 480 = 182400$ while σ_{bu} varies in the range 2.3 (G4) up to 3.7 (G1), with a bias correction of the order of the fraction of kcal/mol in most cases and slightly higher than 1 kcal/mol only for G6, G7, G8 and G1.

In Table II, we report in the second column the vDSSB submitted blind predictions (where the estimate was determined according to the previously cited criteria) along with the Gaussian estimate, the Gaussian mixture estimate (Eq. 7), and the bias-corrected Jarzynski estimate (not submitted). The uncertainties have been computed by bootstrapping with resampling the bound and unbound work samples *prior to* convolution, to avoid an artificial underestimation of the error when resampling from a boosted distribution¹⁵.

In the last column, we report the electrostatic contribution (ΔG_q) to the overall dissociation free energy. In general, we can say that electrostatics is not the driving force for binding. In two cases, the electrostatic balance is unfavorable (G9, G18). For the small G7, most of the binding comes instead from electrostatics, very likely due to the presence of H-bonded water molecules at the entrance of the binding pocket as can be verified from the HREM trajectories provided on Zenodo (<https://zenodo.org/record/5891191>). The convergence of the prediction can be assessed by analyzing the vDSSB estimates, based on the above criteria, as a function of the duration time of the most dissipative process, i.e. the annihilation of the ligand in the bound state (see σ_u and σ_b entries in Table I). From Figure 4, we can see that most of the predictions appear to have converged within the uncertainty, except for G2, G5, G12, G14, and G16. Based on the corresponding σ_{BU} reported in Table I and using $n = 480 \times 360$, the expected bias for $\tau = 1.44$ ns should be less than 0.5 kcal/mol (see Figure 3).

The correlation diagrams and metrics for all the estimates of Table II are reported in Figure 5 and Table III. The large squares refer to the vDSSB ranked prediction. All predictions based on the four estimates are strongly correlated one to the other, whether one chooses the sets including only thirteen guests from SAMPL9, the five additional ligands, and the set comprising all the eighteen ligands. Regarding the agreement with ITC data, we note that, while the Pearson’s and Kendall correlation coefficients R_{xy} are acceptable (for the 13 SAMPL9 guests), good (for all the 18 systems) and excellent (for the 5 extra ligands), most of predictions appear to be systematically

guest	Pred.	Exp.	Gauss	EM(3)	Jarz+bias	ΔG_q
G1	7.8± 0.6	-6.53	6.7 ± 1.1	8.5± 2.6	7.8± 0.6	-0.7 ± 1.2
G2	13.0± 1.2	-10.59	13.7 ± 0.8	13.0± 1.2	14.0± 0.6	1.0 ± 0.3
G3	11.0± 0.8	-8.03	8.2 ± 0.6	11.0± 0.8	11.1± 0.3	0.5 ± 0.8
G4	6.2± 0.8	-6.50	6.2 ± 0.4	7.3± 0.4	7.2± 0.4	1.2 ± 0.4
G5	7.2± 0.6	-5.46	6.8 ± 0.6	7.2± 0.6	7.4± 0.5	5.3 ± 0.2
G6	11.6± 0.4	-8.08	9.2 ± 1.2	11.9± 1.2	11.6± 0.4	0.3 ± 0.8
G7	11.5± 1.9	-7.07	10.3 ± 0.9	11.5± 1.9	12.3± 0.6	11.1 ± 0.7
G8	11.6± 1.6	-6.04	11.5 ± 1.1	11.6± 1.6	12.1± 0.8	2.1 ± 1.5
G9	11.8± 0.9	-6.32	10.4 ± 1.0	12.2± 1.9	11.8± 0.9	-0.5 ± 0.8
G10	12.7± 0.7	-9.96	12.0 ± 0.5	12.7± 0.7	13.0± 0.5	1.2 ± 0.3
G11	8.3± 0.5	-6.26	6.7 ± 0.4	8.3± 0.5	8.4± 0.4	0.7 ± 0.5
G12	16.8± 0.8	-11.02	16.8 ± 0.4	17.5± 0.3	17.6± 0.3	2.8 ± 0.2
G13	6.9± 1.0	-8.58	6.1 ± 0.8	6.9± 0.8	6.9± 0.5	1.5 ± 0.5
G14	14.5± 0.7	9.68	14.5 ± 0.7	15.0± 0.8	15.0± 0.6	5.2 ± 0.1
G15	10.7± 0.6	8.37	10.4 ± 0.5	10.7± 0.6	10.8± 0.6	4.9 ± 0.2
G16	12.9± 0.4	10.59	12.7 ± 0.6	12.9± 0.4	13.0± 0.3	5.1 ± 0.2
G17	8.0± 0.2	6.48	7.5 ± 0.3	8.0± 0.2	8.1± 0.2	2.5 ± 0.2
G18	4.9± 0.4	0.00	4.4 ± 0.5	4.9± 0.4	4.9± 0.4	-3.8 ± 0.3

TABLE II. Estimates of the dissociation free energy in the vDSSB approach. Pred., submitted predictions; Exp., ITC data; Gauss, Gaussian estimates; EM(3), estimates based on Eq. 7; Jarz+bias, estimates based on Eq. 6; ΔG_q , estimated contribution of the electrostatic interactions to the binding (computed using the Gaussian assumption). Energy units are in kcal/mol.

affected by a large *positive* bias [for the binding free energy (BFE)] of the order of 3 kcal/mol. Paraquat (G13) is a notable exception to this trend as the binding energy is in this case *underestimated* by less than 2 kcal/mol. However, this outcome could be due to a cancellation effect, with the prediction being actually much lower than the experimental counterpart when the former is corrected for the systematic and mean signed error.

This molecule was in fact included also in the SAMPL7 challenge for Isaac’s TrimerTrip⁵, and in our SAMPL7 submission paraquat was an outlier, with a strongly *underestimated* dissociation

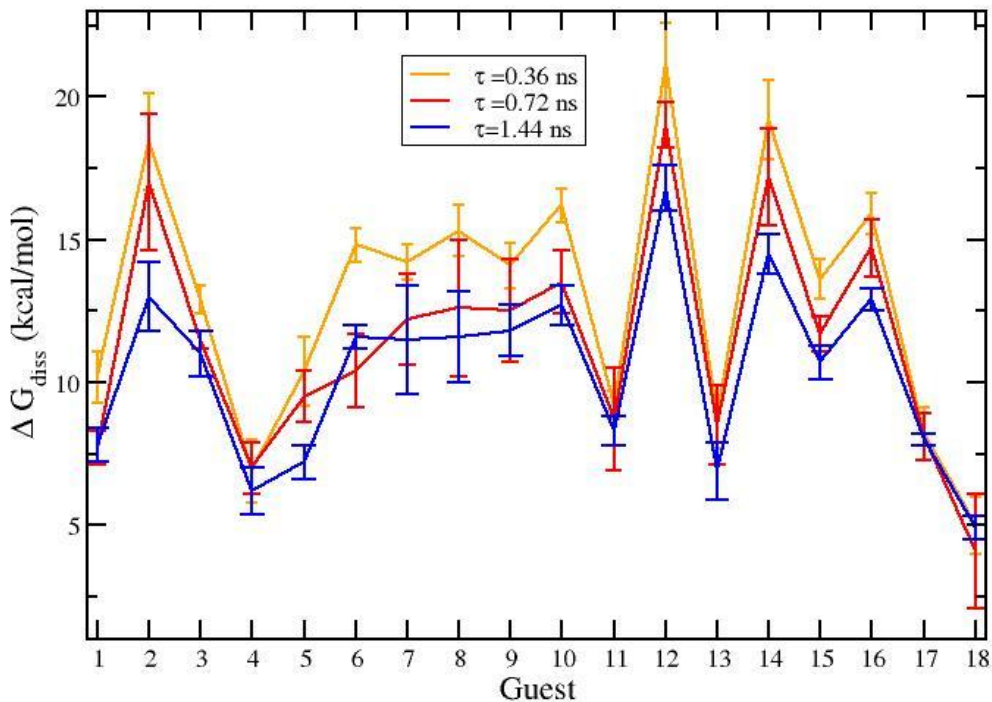


FIG. 4. Convergence of vDSSB estimates of the dissociation free energy as a function of the duration time of the bound state NE alchemical transitions for the eighteen guest molecules reported in Figure 1.

free energy, possibly due to the neglect of polarization in the bound state.³¹ To the light of the structural similarities of WP6 and the TrimerTrip, and as we used exactly the same force field for paraquat in both challenges, we would have expected G13 to be an outlier too in the SAMPL9 challenge. As a matter of fact, if we remove G13 (whose binding affinity was actually known before the submission), the Pearson correlation improves to 0.78 and the τ Kendall to 0.56 while the MUE becomes virtually identical to the MSE ($\simeq 3.1$ kcal/mol, see Table V further on).

IV. DISCUSSION

In Table IV we succinctly describe the five ranked submissions to the SAMPL9 challenge. The Serrillon submission was done using machine learning methods based on a neural network. The He prediction was based on an MM-PB/SA approach (implicit solvent) on conformations generated

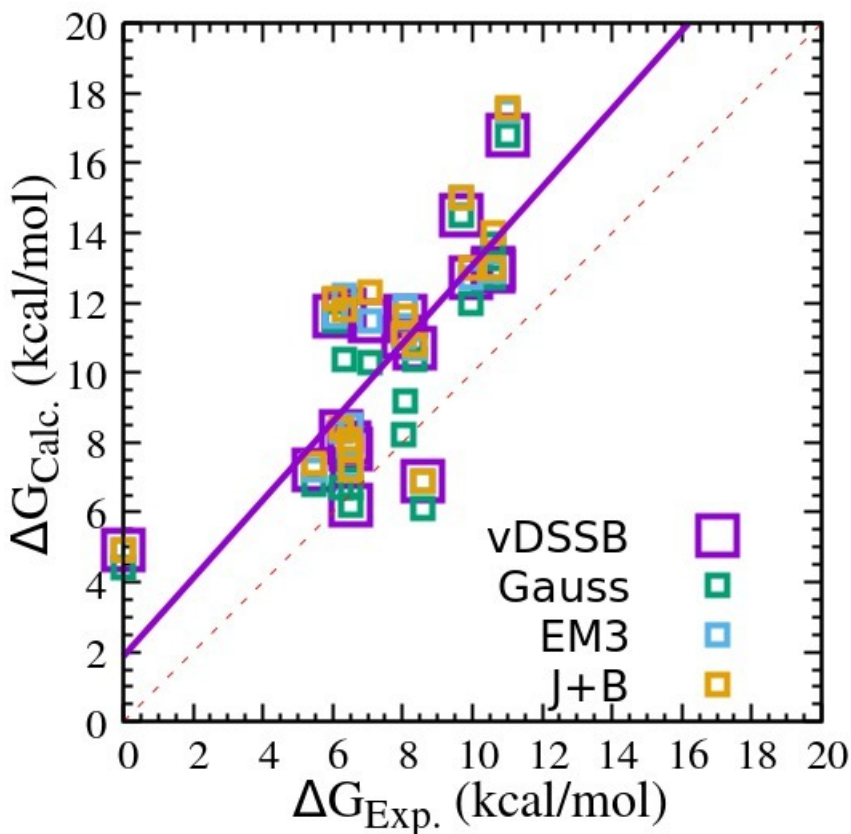


FIG. 5. Correlation diagram for vDSSB-based dissociation free energy estimates reported in Table II

by molecular dynamics with explicit solvent using the GAFF2 force field. We then count three MD-based submissions, using a common alchemical approach each with a different force field, namely the fixed-charge GAFF2/AM1-BCC³⁵ (vDSSB, the present submission), OpenFF2.0⁴⁶ (Voltz) and the polarizable force field AMOEBA⁴⁷ (Ponder). The Ponder and Voelz calculations were done using the traditional free energy perturbation approach with equilibrium simulations for each intermediate alchemical state along the λ -stratification. No enhanced sampling was used in Ponder while in Voelz λ -hopping enforced via a serial generalized ensemble approach^{48–50} with adaptive weights was used (termed “Expanded Ensemble”). In λ -hopping, states can exchange λ values so that a trajectory can visit all λ intermediate states. However, no scaling of the potential energy barriers is enforced, as in e.g. FEP+⁵¹ or in the end-states of vDSSB.¹⁵ The effectiveness of λ -exchanges via HREM was recently assessed in Ref.⁵² where “HREX enhancement [did] not

Est.	R_{xy}	a	b	MUE	τ	MSE	npt
Pred	0.68	1.12	1.87	3.07	0.42	-2.77	13
Gauss	0.70	1.25	-0.10	2.29	0.29	-1.86	13
EM(3)	0.67	1.11	2.18	3.27	0.46	-3.01	13
Jar+B	0.69	1.19	1.66	3.39	0.38	-3.14	13
Pred	0.76	0.93	3.37	3.10	0.50	-2.88	18
Gauss	0.76	0.99	2.19	2.45	0.50	-2.14	18
EM(3)	0.77	0.95	3.49	3.27	0.54	-3.09	18
Jar+B	0.77	0.98	3.33	3.38	0.48	-3.19	18
Pred	0.93	0.84	4.27	3.18	0.80	-3.18	5
Gauss	0.92	0.88	3.70	2.88	0.80	-2.88	5
EM(3)	0.91	0.86	4.24	3.28	0.80	-3.28	5
Jar+B	0.92	0.87	4.25	3.34	0.80	-3.34	5

TABLE III. Precision and accuracy metrics (see text) for the vDSSB estimates of Table II.

Sub.	Method	FF	Restr.	Charge corr.	Neutr.
Voelz	ALCH	OpenFF2.0/AM1BCC	COM(strong)	?	12 Na+
vDSSB	ALCH	GAFF2/AM1BCC	COM (weak)	PME	UBP
Ponder	ALCH	AMOEBA	COM	PME	12 Na+
He	PB/SA	GAFF2/ABCG2	n/a	?	12 Na+/PB
Serrilon	ML	n/a	n/a	n/a	n/a

TABLE IV. Methodological information on the SAMPL9 ranked submissions. ALCH, alchemical; ML, Machine Learning; PB/SA, Poisson Boltzmann, implicit solvent; FF, force field; Restr., host-guest restraint type; Charge corr., correction for charged ligands (finite size); Neutr., Neutralization procedure for the bound state; UBP, uniform background plasma.

appear to give a significant boost to the FEP [Free Energy Perturbation] accuracy”.

At variance with vDSSB where a uniform neutralizing background was adopted, the MD-based Voelz and Ponder used 12 Na⁺ counterions in the bound state. In Table V, we report results of the ranked submissions for the SAMPL9 challenge using as metrics the Pearson correlation coefficient

R_{xy} , the slope a and intercept b of the best fitting line, the Kendall ranking coefficient τ , and the mean unsigned and signed errors MUE, MSE. As far as correlation is concerned, the three MD-

Submission	R_{xy}	a	b	τ	MUE	MSE
Serillon	0.38	0.36	-5.58	0.18	1.56	0.61
He	0.63	0.65	-4.61	0.53	1.89	1.87
Ponder	0.76	1.58	5.28	0.59	1.98	-0.79
Voltz	0.72	1.51	3.25	0.51	2.27	0.68
vDSSB	0.68	1.12	-1.87	0.42	3.07	2.77
Serillon ^a	0.35	0.32	-5.73	0.06	1.56	0.53
He ^a	0.63	0.64	-4.62	0.48	1.92	1.90
Ponder ^a	0.90	1.42	4.61	0.61	1.58	-1.42
Voelz ^a	0.73	1.53	3.35	0.58	2.45	0.73
vDSSB ^a	0.78	1.22	-1.44	0.56	3.19	3.14

^a Metrics computed by eliminating G13 (see text).

TABLE V. Results for the SAMPL9 ranked submissions; Metrics has been. MUE, MSE, and b are given in kcal/mol.

alchemy submissions perform decently, with the Ponder set featuring the best prediction for both R_{xy} and τ and with vDSSB yielding the best fitting line. Ponder is still the best correlated prediction ($R_{xy} = 0.90$) if the G13 guest, with known BFE at the time of the challenge, is removed from the set. The AMOEBA polarizable force field used in the Ponder submission already performed quite well in the SAMPL7 challenge on the Isaac’s TrimerTrip⁵, hence confirming the importance of charge reorganization/polarization in highly polar or charged molecules such as CBn-like compounds or WP6. While the MUE is in the range of [2,3] kcal/mol for Ponder, Voelz and vDSSB, the MSE is positive and large *only* for our prediction, with the majority of the dissociation free energies significantly overestimated. This fact can also be visually appreciated from Figure 6, where the correlation diagrams for the five ranked submissions are reported. Incidentally, we note that the BFE of G13/paraquat (red highlighted symbols) in Serillon (-8.68 kcal/mol), Voelz and He (10.2 kcal/mol) is in accord either with the experimental value using fluorimetric assay reported in Ref.⁷ or with the ITC-determined association constant provided in Ref.⁸. A systematic overestimation

of our vDSSB dissociation free energies was expected, since, before the submission deadline, we found excellent correlation for the five compounds G14-G18 with known ΔG_d^0 but we also found a significant mean MSE (see Table III). In the original submission text file⁵³ we in fact wrote:

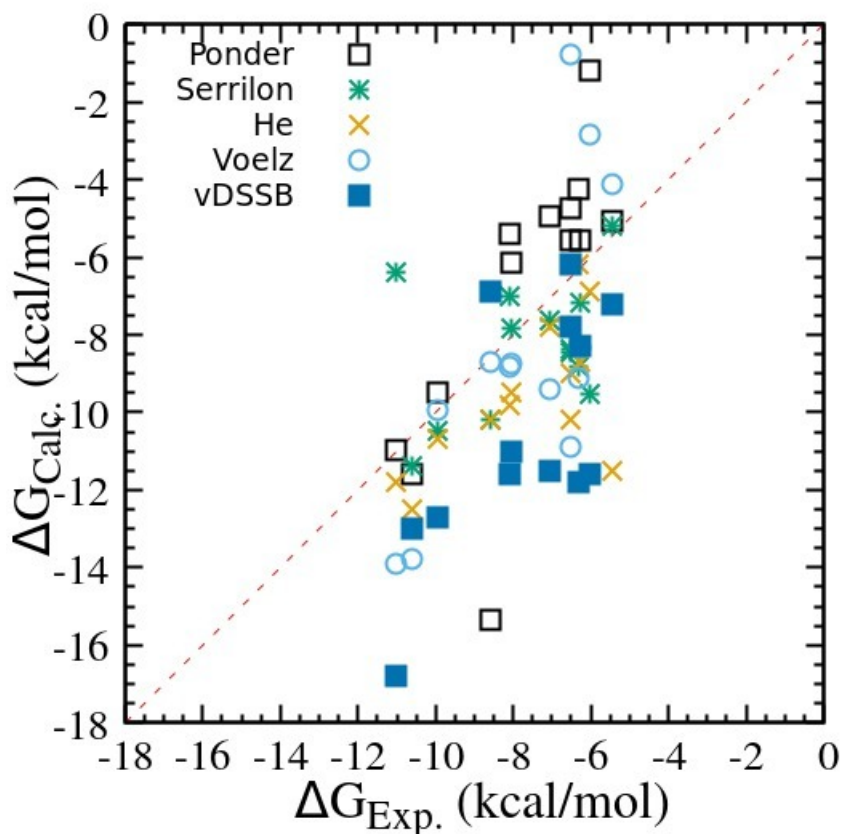


FIG. 6. Correlation diagrams of experimental and calculated binding free energy for the five ranked submissions. The red-highlighted symbols refers to G13 (paraquat).

”given the negative [for dissociation free energy] MSE found in the pre-assessment, we expect a consistent overestimation (in the order of 2-3 kcal/mol) of the submitted BFE, possibly due the presence of protonated WP6 species at $\text{pH}=7.4$ ”. However, such striking discrepancy cannot be attributed to the systematic contribution of complexes with WP6 in different protonation states as the experimentally determined weight of the WP6^{-12} species at $\text{pH}=7.4$ is found to be close to unity. Besides, the large MSE is not observed in the other two alchemy-based predictions. The large MSE in our case is probably caused by the approach used in the neutralization of the MD

box in the bound state. Both in Voelz and Ponder MD-based submissions, neutralization of the 12 negative charges of the host was achieved by introducing an equivalent number of Na^+ counterions. In our prediction, no counterions were used and neutralization was imposed naturally by the PME approach by way a uniform background plasma with constant charge density $\rho = 12/V_{\text{box}}$, using the finite size correction (Eq. 9) for charged guests on the computed ΔG_d^0 . By analyzing the HREM trajectories of the bound state, we found that the WP6 toroidal cavity allocating the guest molecule in most of the cases remains completely devoid of water molecules, hence exhibiting a lower mean dielectric constant than that of the surrounding bulk. In Ref.⁵⁴, Hub et al. showed that, when using a uniform background neutralizing distribution in the context of PBC/PME, artifacts may arise in particular in systems with an inhomogeneous dielectric constant. More in detail, the authors demonstrated that the background charge artificially cause the charged guest to *favor the lower dielectric environment*. The authors provided a simplified correction, based on the Poisson-Boltzmann model, for the free energy difference in the two dielectrics (bulk water and low dielectric environment). For spherical geometry, the correction reads

$$\Delta V = -2\pi \frac{q_{\text{guest}} \rho_{\text{BG}} r_0^2}{\epsilon_l} \quad (12)$$

where ϵ_l is the permittivity of the low-dielectric, r_0 is the radius of the sphere, and ρ_{BG} is the uniform background charge density. The approximated correction Eq. 12 for the spherical geometry cannot be lightly used for the WP6 toroidal cavity with an unknown permittivity, possibly dependent on the guest size and topology. However, we can use Eq. 12 to have an order of magnitude of this correction. In our case, if we set ϵ_l in the range [2,4], $r_0 = [3.5, 4]$ (the effective radius of the WP6 cavity), $\rho_{\text{BG}} \simeq -12/5 \cdot 10^4 \text{ e}/\text{\AA}^3$, and if we use the net charge of the guest for q_{guest} , we find for the thirteen host-guest SAMPL9 dissociation free energies corrections in the range -1.8:3.0 kcal/mol reducing the MUE and the MSE to less than 2 and 1 kcal/mol, respectively, while slightly degrading the Pearson coefficient from $\simeq 0.7$ to $\simeq 0.5$. For the Voelz and Ponder prediction sets these corrections are one order of magnitude smaller as their ρ_{BG} includes *only* the neutralizing charge for the guest molecule being the WP6^{-12} anion neutralized by the twelve Na^+ cations.

Therefore, a Hub-like correction for inhomogeneous dielectric environments in the WP6 complexation indeed explains why our dissociation free energies, obtained with a neutralizing uniform ρ of 12 positive charges, are systematically overestimated while in Voelz and Ponder submissions, where the neutralization was achieved by adding twelve explicit cations, positive and negative

MSE below 1 kcal/mol were found. Moreover, the large Hub correction makes the *dicationic* G13 guest an outlier with an understimation of the dissociation free energy of more than 4 kcal/mol, degrading R_{xy} . Such outcome is consistent with the result obtained for G13 in the case of the SAMPL7 TrimerTrip (characterized by a quasi-toroidal cavity of similar size as WP6) where the dissociation free energy was underestimated by more than 5 kcal/mol using the same force field and a strictly related MD-based alchemical approach.³¹ On the other hand, it should be noted that the vDSSB dissociation free energy of the *chiral* dicationic G12 guest even corrected using Eq. 12, would still be significantly overestimated. In this case, a possible explanation for the discrepancy can be that, as the experimental binding constant refers to the *racemic* mixture⁶, the affinities of the two bulky stereoisomers are disparate and the effective binding constant would be dominated by the *weakest* binder.

Verifying the hypothesis for the consistent overestimation of the dissociation free energy stemming from the uniform background plasma is straightforward but costly. We would need to repeat all HREM and NE calculations for the bound state resizing our MD box using a sidelength of $\simeq 60$ Å to match the experimental Na^+ concentration of 0.14 M⁶, increasing the volume (and the cost) by a factor of four. Besides, convergence of a solution containing diffusing cations and a single heavily charged host-guest complex is certainly harder than that where neutralization is provided by a constant uniform background. In vDSSB, canonical sampling is essential in the end-state for the bound complex. Our HREM scaling protocol, as stated in the Methods section, only involves intra-solute interactions leaving unaltered the solute-solvent balance so as not to accelerate passive diffusion. This is certainly an asset in our approach as the impact on the residence time of the (weakly restrained) guest in the binding pocket is limited but can be an important shortcoming when using explicit counterions by significantly slowing down convergence.

V. POTENTIAL PITFALLS AND WEAK POINTS IN VDSSB

We have previously seen that the estimate type from the work convolution for ΔG_{vdssb} is based on Eq. 6, 7 or on the Gaussian assumption depending on the character of the bound and unbound work distributions as assessed by the AD test or on the uncertainty of the EM estimate. Eq. 6 is used when the AD tests fail and when the EM estimate on the convolution has a large (≥ 2.0 kcal/mol) uncertainty. In this case Eq. 6 is corrected for a theoretical σ and n dependent positive bias computed from a Gaussian distribution with the same variance of the convolution

work distribution. If the latter departs significantly from a normal distribution, the bias can be overestimated with an impact on the accuracy of ΔG_{vdssb} .

A weak point of *any* alchemical approach, including vDSSB, is related to the standard state correction, connected to the elusive binding site volume^{31,55-57}. In vDSSB, where a weak COM-COM restraint is used, the *negative* correction to the *dissociation* free energy is estimated from the variance of the host-guest COM-COM distance distribution in the bound state and is hence a characteristic of the host-guest complex, varying in a range -5:-2.5 kcal/mol (see Table I). In principle, the binding site volume should depend on both the host and the guest in the context of the NE alchemical theory²⁹. In Voelz and Ponder FEP calculations, the volume correction is related to the force constant of the host-guest COM-COM restraint as $V_{\text{restr}} = (\frac{2\pi RT}{k})^{3/2}$ and, as such, is *identical for all guests*, in the assumption that in the simulation of the restrained complex all states that are made accessible by restrained potential have been canonically sampled for all λ states along the alchemical path^{55,57}. Such correction to the dissociation free energy is equal to -3.85 in Voelz⁵⁸. In Ponder, again an *identical* volume correction was used for all guests using COM-COM restraints defining a flat-bottom potential “chosen such that [these restraints] were not violated during unrestrained simulations runs on the bound host-guest complex.”⁵⁹ The size of the Ponder standard state correction was not given in Ref.⁵⁹. If we remove our guest-dependent standard state volume correction (ΔG_{vol} entry in Table I) assuming an identical correction of -4 kcal/mol for all guests as in Ponder and Voelz, agreement with experiment luckily improves, yielding $R_{xy} = 0.84$, $\tau = 0.49$, $a = 1.60$, MUE=2.18 kcal/mol and MSE=1.50.

VI. CONCLUSION

We have presented our results on the SAMPL9 challenge focusing on binding of soluble carboxy-pillar[6]arene)with ammonium/diammonium cationic guests. We adopted an alchemical technique, named virtual double system single box, relying on an enhanced sampling of the end-states and on the production of few hundreds of fast nonequilibrium trajectories where the ligand is decoupled and recoupled in the bound and unbound state, respectively. The resulting work distributions were combined (convoluted) as if the two independent processes occurred in the same box, with one ligand on the host and the other in the far distant bulk. The dissociation free energy was estimated from the convolution using the Jarzynski theorem, the Gaussian assumption, or the Gaussian mixture assumption, depending on the character of the bound and unbound work distributions.

Correlation with experimental data is acceptable and consistent with our previous SAMPL6 and SAMPL7 submission also based on nonequilibrium alchemy. If G13 (paraquat), whose experimental binding constant was known at the time of the challenge⁵³, is removed from the set, vDSSB is the second best-correlated submission of the challenge (see Table V). Nonetheless, when compared to the other two MD-based SAMPL9 alchemical submissions, our prediction set for the dissociation free energy exhibits a large mean signed error (MSE), suggesting a systematic error in the simulation protocol. We attribute the large MSE to the way neutralization was imposed in the bound state containing the WP6⁻¹² anion and the guest. At variance with the other MD-based submissions, where explicit counterions were introduced, we used a uniform neutralizing background plasma. The uniform background charge approach has been recently demonstrated to artificially favor the binding in the lower dielectric environment.

Overall, the challenge has confirmed that MD-based alchemical methods tend to correlate better with experiment with respect to other approaches (e.g. Machine Learning or Poisson-Boltzmann with MD sampling of the end-states). However, accuracy is still falling short in MD protocols, with a mean deviation of the order of 2 kcal/mol even when resorting to computationally demanding polarizable force field. In this regard, we note that the present SAMPL9 challenge (as well as some of the past challenges) is characterized by exceptionally critical issues from the parameterization perspective mostly related to electrostatic interactions, such as unknown protonation state of the highly charged host molecule, chiral specificity in binding, singly or doubly charged guests. Besides, in most of the recent SAMPL initiatives, all guest molecules share in general a common chemical structure, characterized by charged substituents on a hydrophobic scaffold while the hosts are rigid macrocyclic molecules heavily decorated with polar or charged groups. As recently shown in Ref.⁶⁰, small imperfections on the electrostatic modeling can have a huge impact on the BFE. Possibly, these imperfections could be systematically enhanced when testing MD-based approaches with “general” electrostatic parameterizations in the recent SAMPL challenges.

SUPPORTING INFORMATION

The bound and unbound state work distributions for all eighteen ligands of Figure 1 are reported in the Supporting Information. PDB trajectory files, raw work data, and force field parameter files are available at the general-purpose open-access repository Zenodo (<https://zenodo.org/record/5891191>).

The ORAC program (v6.1) is available for download under the General Public License at the

website <http://www1.chim.unifi.it/orac/>

Third-party software Autodock Vina can be downloaded from the website <https://vina.scripps.edu/>

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