Ref: Dual Effect of Secondary Solutes on Binding Equilibria: Contributions from Solute–Reactant Interactions and Solute–Water Interactions; http://doi.org/10.1021/acsomega.3c09329
Corresponding Author: Daryl Eggers

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AUTHOR RESPONSE TO REVIEWS FROM *J. PHYS. CHEM. B*

Submission ID: jp-2023-06736u

We thank the *JPCB* reviewers for their critiques. Our responses appear below, indented in blue font, following the specific comments of each reviewer. Changes to the manuscript for *ACS Omega* submission are found in a separate "File not for publication" with the changes highlighted in yellow.

Reviewer: 1

Recommendation: This paper is not recommended because it does not provide new physical insights or develop new methods.

Comments:
In this manuscript, the authors explore the influence of various cosolvents (which they term "secondary solutes") on association reactions. To this end, the authors combine results of isothermal titration calorimetric measurements with their previously developed theoretical framework. Despite the importance of the topic, I cannot recommend publication of the manuscript. Instead, I recommend its rejection, since the theoretical framework used in the manuscript is deeply flawed. It is based on vague and poorly justified assumptions and, in some cases, contradicts the fundamentals of thermodynamics.

(i) Eq. (8) is the main equation of the manuscript. It is based on Eq. (1) which had been derived by the authors previously in Ref. (2). It is based on two false premises. Firstly, it is assumed that the number of water molecules which are exchanged between the bulk and hydration phases upon an association reaction, n, is equal to the number of water molecules hydrating the product. This arbitrary assumption is certainly incorrect.

> This assertion is wrong. We make no assumption about the number of water molecules involved (n). We only stress the fact that there are fewer water molecules in contact with the binary complex than bound to the surfaces of the two (free) reactants when they are

not associated with each other. This must be true because the association of two molecules will always remove some of the surface area in contact with the solvent. Our governing equation acknowledges that the number of molecules released is stoichiometric with the number of complexes formed, but it does not equate "n" with the total hydration sphere of the product, as stated by Reviewer 1 above. On the contrary, we expect much of the water initially bound to the reactants to remain bound when the complex is formed. The actual number of water molecules involved for each model reaction is unknown but embedded in the value of ΔG^S (Eq.3).

Secondly, the derivation of Eq. (1) is based on a statement that "the chemical potential of water is a complicated function of hydrogen-bond strength and molecular orientation and not a strong function of the water concentration or number density" (Ref. 2). The statement is wrong; the authors may want to re-read the definition of chemical potential in any of many Physical Chemistry textbooks.

The statement in question is meant to convey the idea that enthalpy and entropy changes in water are more (energetically) important than changes in density (PΔV). One need only view the molecular structure of ice to appreciate two of the extremes a water molecule may adopt in the liquid state, one of most favorable enthalpy (a maximum number of perfectly-oriented H-bonds) and one of least favorable entropy (a hexagonal lattice). Presumably, solutes can induce all possible combinations of enthalpy and entropy, depending on the chemistry of the solute at the interface. Because Reviewer 1 is referring to a statement in a previous publication, it has not prompted any changes to the current manuscript.

Furthermore, in Ref. 2, the authors talk about "the free energy per mole of a specific subset of water molecules"; since "free energy per mole" corresponds to chemical potential, the authors state that the chemical potentials of different subsets of water molecules within the same solution can be different! This misconception is also used in multiple places of the current manuscript (see below). (and see author response below)

In short, Eq. (8) is physically incorrect and cannot be used for analysis of experimental data.

(ii) Along the same lines, the solvent-associated term in Eq. (8) is defined as the product of the hydration number and the difference in free energy of bulk and hydration waters normalized per mole of water. Again, these values correspond to chemical potential which, in equilibrium, must be the same for all water molecules either within the hydration shell of a solute or in the bulk.

We humbly disagree. When the system is at equilibrium, there can be no change in energy of the system when a water molecule moves from one position to another position (for example, from a solute surface to the bulk). This requirement does not imply that a specific water molecule cannot experience a change in free energy; it only implies that the change in energy of the selected water molecule must be compensated by another water molecule that moves in the opposite direction, from the bulk to the surface. As stated in our Discussion section, we agree that the time-averaged chemical potential of all water molecules in the solution are equal at equilibrium, but the location-specific potentials depend on the presence (and chemistry) of adjacent surfaces. This realization is at the very core of our thermodynamic framework; we treat all solutes and surfaces as defining a boundary condition that may alter the chemical potential of neighboring water molecules. Please also note that the free energy terms for water in our equations are normalized to the moles of water *in each distinct subpopulation*, either

the bulk or hydration shell, as explained under Eq.3 where we refer to their "location-averaged values."

Much of this response has been appended to the second paragraph of the Discussion. We thank Reviewer 1 for prompting us to clarify a very important point regarding solution equilibria.

(iii) In Eq. (6), the term RTln([AB]°/[A]°[B]°) is, by definition, equal to zero.

This is true if the standard state concentration is defined as 1.0 M for each reactant, but our approach is not limited to this definition. In fact, we suggest that the standard state concentration should be re-defined as an infinitely-dilute solution in our previous publications (Refs. 1-2). It has never made sense to us to set 1.0 M as a standard state concentration when many compounds are not even soluble to such a high concentration in water, and when some compounds are so large (proteins, for example) that a 1.0 M solution would require atoms to overlap! The constant term cited in item (iii) above is embedded in the value of ∆G° regardless of the standard state definition, so Eq.6 is unchanged for this revised manuscript.

(iv) In many places, the authors use the undefined term "thermodynamic activity". For example, on p. 24, they write state that "a solution may contain coexisting subpopulations of water that differ in thermodynamic activity". On p 26, they write "…the subpopulation of water molecules located next to each boundary may have a significantly different thermodynamic activity than the bulk (time-averaged) value". Apparently, the authors mean chemical potential; but, as explained above, this violates laws of thermodynamics.

This is a valid criticism. Our thermodynamic framework does not employ "water activity" as one of the parameters because it would require a different activity coefficient for every possible surface chemistry! The revised manuscript now substitutes the phrase "chemical potential" or "free energy" in every instance where we referred to water activity in the previous draft.

On p. 35, the authors talk about "the thermodynamic activity of charged and polar groups found on the surface of a folded protein". This statement is quite paradoxical; I do not know what "the thermodynamic activity" of a particular group may mean.

We have changed our wording to "increase the chemical potential of the protein at surface residues corresponding to polar and charged groups" in the cited paragraph. To us, it seems reasonable that the chemical potential of a large macromolecule should be some additive function of the many chemical groups that define its boundary with the solvent.

(v) The speculations about the origins of the hydrophobic effect on pp. 30-31 are poorly unjustified and rather perplexing. Apparently, the hydrophobic effect has nothing to do with water!

We expect our discussion of the hydrophobic effect to be unsettling for many scientists. But, in an attempt to summarize the main points in more-agreeable terms, our work simply "moves the bar" separating favorable from unfavorable desolvation energy such that hydrophobic groups are near the zero point. We also emphasize that all changes in solvation energy must be calibrated to the free energy of the bulk phase (which can be altered by the presence of a cosolute). This concept is nicely depicted in Fig.5 of Ref.4.

In short, the analysis of experimental data is based on a flawed theoretical basis and un-thermodynamic assumptions.

Additional Questions:
Significance: Moderate (not suitable for The Journal of Physical Chemistry)
Originality: Moderate (not suitable for The Journal of Physical Chemistry)
Broad Appeal: Moderate (not suitable for The Journal of Physical Chemistry)
Clarity: Low (not suitable for The Journal of Physical Chemistry)
Are the conclusions adequately supported by the data?: No
Does the paper supply significant physical insight and/or a new method of general interest?: No
Does the paper provide sufficient experimental and/or computational data and details to allow for reproducibility of results?: Yes

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Reviewer: 2

Recommendation: This paper is not recommended because it does not provide new physical insights or develop new methods.

Comments:
I have read the manuscript by Eggers and colleagues, and also the Supporting Material and the Editorial by prof. White, Editor of Supramolecular Chemistry: I am really astonished because this has been dragging on for ten years (Eggers and Castellano published the first article on this matter in J.Phys.Chem.B 2013, 117, 8180).

The authors claim that the well-established procedure to analyze experimental data of binding equilibria in aqueous solutions to obtain right thermodynamic values is not correct; it would be necessary to add an extra Gibbs free energy contribution accounting for the effect of water molecules that are released from the first hydration shell of the two molecules upon forming the non-covalent complex. The need is because they obtain, by means of ITC measurements, values of the "apparent" equilibrium constant that depend on the concentration of reactants in highly non-ideal solutions. It is well-established that, in the presence of ionic species, such as calcium ions and EDTA, the effect of charge-charge interactions leads to non-ideal behavior also for very low molar concentrations; look at the Debye-Huckel theory and its strong limitations. So there is no surprise in the experimental results.

> The phrases "apparent equilibrium," "true equilibrium" and "non-ideal behavior" are terms invoked by physical chemists when their results do not match the expectation of classical thermodynamics. But what if the non-ideal behavior of ionic species, like binding of Ca^{2+}/EDTA, is the outcome of omitting a term that accounts for changes in the chemical potential of water? And how does one explain the concentration dependence of binding models that do NOT involve strong ionic interactions (as demonstrated in the *Supramol.Chem.* paper, Ref. 4)? We are asking the scientific community to keep an open mind when evaluating our approach because current practices fail to answer many questions in aqueous solution thermodynamics, some of which are listed in Ref. 3 by Paul Cremer, et al.

The claim by Eggers and colleagues could be considered valid solely if they are able to show that the values of the true equilibrium thermodynamic constant (calculated accounting for non-ideal effects) depend upon concentrations of the reactants.

Again, "true equilibrium" is a phrase that was created to defend the classical approach. We view the experimentally measured equilibrium ratio as the "true equilibrium ratio," though we have made it clear that linked equilibria may contribute to the observed thermodynamics (Eq.2 of current manuscript and Supplemental Data file in Ref. 4).

Finally: (1) it seems that Eggers and colleagues use always the same examples and this year have already published this "thing" in Supramolecular Chemistry;

We thought it was logical and appropriate to use the same binding models that were tested in the past (in dilute solutions only) when expanding our studies to the presence of cosolutes. All of the data obtained in urea, sucrose, trehalose, and phosphate solutions are original, unpublished results. Also, we would like to mention that the desire to understand the effect of cosolutes on binding and conformational equilibria has been the long-term goal of the corresponding author's research program; this paper is the culmination of ~25 years of thought and effort.

(2) Gilson and colleagues have already published in BBA 2018, 1862, 692 (reference 23 of the manuscript), a similar criticism on the claim advanced by Eggers and colleagues.

The critique by Gilson and colleagues was addressed in the reviews of our recent paper (Ref. 4), as published in the shared editorial. Also, please see our response to item (ii) under Reviewer 1 above, which overlaps with the criticisms of Gilson.

Two final points: (a) the text contains several sentences starting with "we propose";

The phrase "we propose" was replaced in two locations of the Discussion with a more appropriate wording.

(b) statistical mechanical arguments must be used to advance a "new" expression of the chemical potential of a solute in water or aqueous solution.

We have not changed the expression for the chemical potential of a reacting solute in water (see Eq.3 of Ref. 2, for example). Our framework differs from the conventional approach by giving explicit consideration to the change in water potential that accompanies a (solvent-balanced) binding reaction.

It is our sincere hope that our work will inspire a few open-minded scientists to develop new computational approaches for assessing the chemical potential of water as a function of position and to incorporate changes in solvation energy in their final analyses.

Additional Questions:
Significance: Low (not suitable for The Journal of Physical Chemistry)
Originality: Low (not suitable for The Journal of Physical Chemistry)
Broad Appeal: Low (not suitable for The Journal of Physical Chemistry)
Clarity: Low (not suitable for The Journal of Physical Chemistry)
Are the conclusions adequately supported by the data?: No
Does the paper supply significant physical insight and/or a new method of general interest?: No
Does the paper provide sufficient experimental and/or computational data and details to allow for reproducibility of results?: Yes

Reviewer: 3

Recommendation: This paper is publishable subject to minor revisions noted. Further review is not needed.

Comments:
Eggers and co-workers report a thermodynamic study on the role of water in host-guest binding. The manuscript builds on their recently published study (Supramol. Chem. 2023) and treats the effects of co-solutes that alter water structure.

The work is provocative from the opening paragraph, stating "the classical relationship for binding equilibria in solution must be viewed as originating from an unbalanced reaction." The proposed imbalance arising from the unaccounted water molecules. Some of the conclusions are also provocative (controversial even), e.g. "the energetic contribution of the released water molecules that accompany a nonpolar–nonpolar interaction is negligible."

Overall the paper is robust and the data are soundly interpreted. The discussion is perhaps overly long, in particular, "Other Cosolute-Related Issues". One criticism concerns the use of different guests for macrocycle complexation. Selecting one guest could enable better comparison. Benzylamine, a crude analogue of phenyalanine could be interesting. (If necessary, n-benzylacetamide could be tested, lower affinity for CB7).

> We thank Reviewer 3 for the positive comments and suggestions. We agree that the Discussion section is long, but we are hesitant to remove any content because we view all of the discussion as relevant to understanding how our approach differs from the past. Regarding the use of benzylamine for testing multiple host molecules with the same guest molecule, that is an interesting research direction for a future project.

Amino-glucose binds CB7 (Chem. Eur. J. 2016, 22, 15791-15799) as proven by X-ray crystallography (CCDC CAFJEB). Notwithstanding the contribution of the amine, it is conceivable that 1 M sucrose will result in CB7 complexation. So, could sucrose be competing with AcPheNH2 complexation resulting in an apparent reduced affinity for the latter? Furthermore, "clustering" was invoked in the case of BCD. Clustering is important also for CB7, see Angew. Chem. Int. Ed. 2018, 57, 7126-7130.

> We have read the two cited references above, and we believe sucrose is unlikely to bind CB7 with any measurable affinity because (a) the protonated amine group is crucial for binding of the amino monosaccharides in the reference paper, and (2) sucrose is a disaccharide that is larger in molecular volume than the cavity of CB7, about 360 $Å^3$ to 280 $Å^3$ for the cavity.

> Regarding the clustering of CB7, it appears that most of the evidence is inferred from samples in the solid state where CB7-CB7 contacts may be prerequisite for crystal formation. Interestingly, we did find a paper that demonstrates CB7 can form a hydrogel, but the interactions responsible for gel formation are mediated by hydronium ions at pH 0-2 (see *Angew. Chem.* 2007, 119, 214–217, by Hwang, et al.).

Minor comments:
Consider broadening the scope by changing the opening sentence to include "the (bio)physical sciences."

> Done.

Scheme 1 may work better if all three macrocycles are represented using the shorthand of a bracketed monomer, as used for BCD. The current CB7 representation is not ideal.

> Agreed. The macrocycles are now more uniformly presented as bracketed monomers.

Is AcPheNH2 the correct shorthand for N-acetyl-phenylalanine-amide? The –NH2 portion suggests amine, not amide.

> Yes, AcPheNH$_2$ is the accepted shorthand, and this is how the manufacturer labels the reagent on their bottle. The α-amino group is always assumed to be left of the amino acid abbreviation, Phe, and the α-carboxyl group to the right. Thus, -NH$_2$ on the right side of the abbreviation denotes an amide linkage.

It would be appropriate to cite at least one paper from Urbach, who has amply demonstrated CB7 complexation of Phe.

> A reference to Chinai, et al., *JACS*, 2011, has been added.

Regarding the hydrophobic effect as "driven by the release of high-energy water" consider citing also the work of Bender (ref 27).

> A reference to Griffiths and Bender, *Advances in Catalysis*, 1973, has been added.

Reconsider the use of "Disappointingly," which suggests an emotive involvement in an otherwise objective study!

> Agreed. "Disappointingly" has been replaced with "However" in the revision.

What is meant by "the interstitial space between solute molecules"? Are interstices relevant to solutes at low concentration in water?

> We have replaced "interstitial space" with "intervening space," in reference to the solvent volume that is not influenced by a boundary condition. Our main point is that a given water molecule will sample all possible locations in a solution over time.

Additional Questions:
Significance: High (suitable for The Journal of Physical Chemistry)
Originality: High (suitable for The Journal of Physical Chemistry)
Broad Appeal: High (suitable for The Journal of Physical Chemistry)
Clarity: High (suitable for The Journal of Physical Chemistry)
Are the conclusions adequately supported by the data?: Yes
Does the paper supply significant physical insight and/or a new method of general interest?: Yes
Does the paper provide sufficient experimental and/or computational data and details to allow for reproducibility of results?: Yes

> We thank Reviewer 3 for the constructive comments which have led to significant improvements in the content and readability of our manuscript.

AUTHOR RESPONSE TO REVIEW FROM ACS OMEGA

Submission ID: ao-2023-093297

Reviewer: 1

Recommendation: Publish after major revisions.

Comments:

Eggers, et al. present an intriguing extension of previous thermodynamic models for binding events to include explicit contributions from water molecules whose energetics change upon desolvation due to binding. Overall, the work is well-presented and an important contribution to the field. There are two issues that need to be resolved, in my view, prior to publication.

1. Figure 5 presents fits with zero slope, yet there is no statistical justification for this deviation from the linear fits used for all other measurements in the study. A statistical justification should be included by fitting these data sets to both the zero slope and the linear model with a comparison of reduced chi-squared values. The choice of the zero slope model as the model for best-fit should be supported by a failure of the more complex linear model to improve the reduced chi-squared value more than a few percent. I suspect such an analysis will support the authors' choice of the zero slope model, but a statistical comparison is essential, particularly given the somewhat radical interpretation of the zero slope results.

> Figure 5 has been revised to show the slope of the trend line for the top three datasets in the graph. The linear fits were not as bad as we originally perceived (0.79 was the lowest value). For the control dataset in dilute solution, the zero-slope model was maintained because the linear fit is just 0.08. We now include the R^2 values for all model binding reactions in Table 1, and the discussion section has been updated to reflect these changes.

2. The authors generalize the results for the β-cyclodextrin data (Figure 5) inappropriately (page 31). In discussing these data, the authors make an argument that the widely supported model for the hydrophobic effect being driven by changes to the energetics of water molecules is flawed. The authors state in their discussion, "This line of reasoning leads us to suggest that the hydrophobic effect is better described by the phrase 'aqua indifferens effect.'" They elaborate on this statement to imply that the apparent zero-energy-change supported by the β-CD/-AC data represents a general misconception in the field, citing a single molecular dynamics study as corroboration of this conclusion. While I agree that the data presented suggest an energetically neutral desolvation event, the literature on hydrophobic hydration clearly establishes that the nature of hydrophobic hydration is dependent on size, curvature, and topology in a complex fashion. It is unreasonable, and a vast overinterpretation, to generalize the presented finding as representative of the typical energetics for hydrophobic hydration (or dehydration). The authors should revise this discussion with more thorough citation of the literature and a more measured discussion of this result.

> This comment prompted a new figure for the discussion, as reproduced below. Our "alternative view" of the hydrophobic effect does not change anything related to studies on surface hydration as a function of chemistry, geometry, etc., when comparing one surface to another surface. As described in the figure legend, we are merely suggesting that the reference point, the free energy of water in the bulk phase, must have a set value which we believe is higher than normally perceived. Because we are currently the only research group that includes bulk water molecules in the thermodynamic framework

for analyzing binding equilibria, we are the first to estimate (experimentally) the change in solvation energy that accompanies the binding of two hydrophobic surfaces.

Regarding the small sample pool on which our hypothesis is made, we have inserted a more impartial statement in the Conclusions and in the Result section for βCD. For example, in the paragraph that precedes Fig. 5, we now state the following:

"Small changes in solvation free energy appear to be a general feature of nonpolar interactions, though few examples have been characterized by this approach."
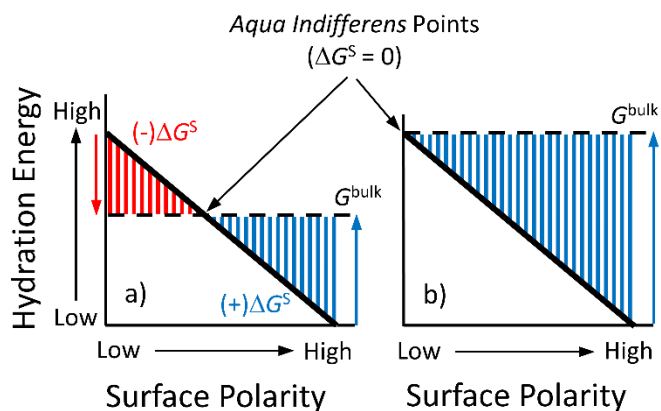
Figure 6. Raising the bar for bulk water in defining the change in solvation free energy. (a) Common view of solvation free energy for which the release of water from nonpolar solutes and surfaces is negative and favorable (red region) and release from polar solutes and surfaces is positive and unfavorable (blue region). At some intermediate point in polarity, there must exist a surface that corresponds to $\Delta G^S = 0$, labeled here as the *aqua indifferens* point. (b) Alternative view of solvation free energy in a dilute solution for which the value of G^{bulk} is raised (horizontal dashed line) until the change in solvation free energy for a surface of low polarity (*i.e.*, hydrophobic surface) is near the *aqua indifferens* point. The ranking of hydration energies as a function of surface chemistry (diagonal line) is the same for both panels.

As a final point of clarification, the final sentence of the first paragraph on page 26 of the manuscript should be revised to improve clarity. The sentence reads, "Consequently, addition of a secondary solute will always shift the bulk water free energy toward the energy of the cosolute's hydration sphere, influencing the thermodynamics of any reaction in that defined solution." I think the authors' meaning here is to say that they are including the hydration sphere of the cosolute in the pool of 'bulk water' energetically, thereby simplifying the thermodynamic treatment of the water involved in the binding event (i.e. NOT treating any exchange of water between the true bulk and the solvation shell of the cosolute explicitly). If my interpretation on this point is correct, I agree that this treatment makes sense. If it is not, then the lack of clarity is made evident. In any case, this section of the manuscript would be improved with a more deliberate description so as to make the logic behind this treatment abundantly clear.

Reviewer 1's interpretation is correct in assuming that we define bulk water as including the hydration sphere of the cosolute. To clarify this point, the following statement was inserted in the Introduction section following Equation 3:

> *"Using set theory terminology, bulk water is defined here as the complement to the solvation spheres of the reactants; bulk water includes the solvation spheres of all (nonreacting) cosolutes, in addition to all water molecules that are distant from any surface."*

Regarding the statement in the paragraph that was questioned by Reviewer 1, a minor change in wording has been made. The phrase "*will always shift the bulk water free energy*" has been altered to "*will always shift the average free energy of the bulk phase.*" We hope these revisions are adequate to avoid confusion by other readers.

Additional Questions:

Is the technical quality of the research reported within valid and appropriate?: Yes
Please evaluate the degree of novelty and originality of the research reported: Excellent
Are the conclusions adequately supported by the data presented?: Yes
Are the literature references appropriate and up to date?: Yes

We thank Reviewer 1 for their time and thoughtful comments!