Osmolyte depletion viewed in terms of the dividing membrane and its work of expansion against osmotic pressure

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**ABSTRACT**

How osmolytes enhance the folding, binding, and self-assembly of biological macromolecules at a microscopic scale has long been a matter of debate. Ambiguities persist on the key interpretive concepts, such as the “effective membrane” (which marks the boundary of the volume from which osmolytes are excluded) and the “free energy of exclusion” of osmolytes from biomolecular surfaces. In this paper, we formulate these elusive concepts based upon chemical thermodynamics and rigorous statistical thermodynamics (the Kirkwood-Buff theory). Positioning of the membrane at the osmotic dividing surface is crucial in order not to affect the thermodynamics of solvation. The notion of the free energy (work) of excluding osmolytes is refined to the expansion work against the osmotic pressure, which indeed describes the change of solvation free energy at dilute osmolyte concentrations.

**1. Introduction**

Self-assembly is important in a wide range of scientific disciplines, from biochemistry (protein assembly and self-aggregation)[1-8] to nanoscience and colloid and interfacial science (self-assemblies and surface forces) [9-14]. Self-assembly is caused by the attractive forces between the constituent macromolecules, which cannot be understood quantitatively without a consideration of the surrounding water and cosolvent molecules that mediate these forces [1-16].

When considering how cosolvents work to enhance self-assembly, we are faced with a variety of synonyms for cosolvents (e.g. solutes, cosolutes, hydrotropes, solubilisers, additives, denaturants, stabilisers, kosmotropes, chaotropes, or osmolytes) which has brought further complications [1-7,15]; here we define “cosolvents” as the third component in general, and “osmolytes” for a particular class of cosolvents, which are involved in the stabilization of the native protein structure, protein-ligand interaction, as well as self-assembly [1-8,15,16]. A clear, molecular-level understanding of the role of osmolytes will have a fundamental and far-reaching significance not only in a wide variety of scientific disciplines but also in applications (such as in formulation science), where the right choice of cosolvents makes a drastic difference in solvent-related processes [17-24].

So how, at a molecular level, do osmolytes enhance the folding, binding and self-assembly of macromolecules? The following two hypotheses have coexisted for decades [1-10]:

1. the **depletion forces** hypothesis, which attributes the enhancement of association to the exclusion of osmolytes from biomolecules, colloids, or surfaces [3,4,6-8,13,14,17];
2. the **hydration forces** hypothesis, which attributes the enhancement of association to the change of hydration induced by the presence of osmolytes [2,5,9,10,15].

Are these two hypotheses equivalent or contradictory? This has indeed been a very difficult question to answer from a molecular basis [1-5,25-28]. The reason is twofold: (i) unlike protein-ligand binding or the self-assembly of biomolecules, protein-osmolyte interactions are weak and non-specific [1-7,25-28]; (ii) osmolytes act on proteins not by binding but by depletion, i.e., being preferentially excluded from proteins [1-7,25-28]. Yet the early development of biomolecular thermodynamics focused mainly on specific interactions; this has made it challenging to describe weak, non-specific, depletion interactions based upon the stoichiometric binding models of specific interactions [25-28]. These seminal earlier theories [2-5,25-28] remained purely phenomenological and approximate, until only recently the rigorous statistical thermodynamic re-derivation, based upon the Kirkwood-Buff (KB) theory, has finally brought a clear description of weak, non-specific interactions [6,7,19,20].

The assumed equivalence between the two has led to the osmotic stress technique (OST), i.e., the use of the osmolytes for probing hydration changes that accompany protein-ligand interactions, allosteric effects, and ion channel openings [2,5,9,10,15]. The basic assumption of OST is that the addition of osmolytes somehow exerts osmotic pressure on hydration water molecules that are located in the vicinity of biomolecules [5]. This approach, despite its widespread use, has also been a cause of debate over the last few decades [1-7]. Using a rigorous KB theory, we have shown that the exclusion of osmolytes from biomolecular surfaces is the origin of the osmolyte-induced equilibrium shift [6,7]. This conclusion, which supports the depletion hypothesis, is the rigorous theoretical endorsement of the macromolecular crowding theory [29,30], yet is at odds with the hydration hypothesis [2,5,9,10,15] which assumes the equivalence between osmolyte exclusion and biomolecular hydration.

However, we believe that the theoretical basis for the presumed equivalence between hydration and depletion should be revisited from a rigorous statistical thermodynamic perspective, as it can provide an alternative interpretive approach on preferential solvation. The previous controversy over the equivalence of depletion and hydration can be summarised into following four main points [1-7]:

1. **Introduction of an effective or hypothetical semi-permeable membrane**, which separates the biomolecular vicinity from the bulk solution [3-5].
2. **Free-energy change of the system due to the osmotic pressure** arising from the inaccessibility of the osmolytes [5,9], which persists even in the absence of the semi-permeable membrane, due to osmolytes’ steric inaccessibility [5,9].
3. **“Free energy of exclusion” of osmolytes from biomolecules** as the driving force of macromolecule-macromolecule and surface-surface association, because association reduces the work required to exclude osmolytes [3,4].
4. **Pressure-volume work of excluding osmolytes** can be attributed solely to the change of biomolecular hydration [5].

Ambiguity persists in (I)-(IV), especially when the membrane was employed explicitly in the experimental setup, sometimes it was deemed superfluous[5] yet was nevertheless employed conceptually in the interpretation [3-7]. What is even more puzzling is that the osmotic pressure is assumed to arise from osmolytes’ inaccessibility to the hydration shell, even when there is no real semi-permeable membrane separating the hydration shell (vicinity) from the bulk (this is how the hypothetical “effective membrane” has been introduced to the system [3-5]). Therefore, the first aim of our paper is to develop a rigorous statistical thermodynamic theory, in order to clarify what the “effective” membrane really does to the osmolyte-induced shift of biomolecular equilibria, thereby clarifying (I) and (II) as summarised above.

By introducing the “effective” semi-permeable membrane explicitly into our theory, we will be able to address our second aim, i.e., is to examine the validity of another elusive concept, the “free energy of exclusion” of osmolytes from biomolecules, as summarised by (III) and (IV) [3,4]. Is the work of osmolyte exclusion really the change of biomolecular solvation free energy? The fact that this question has been addressed only phenomenologically and intuitively has perpetuated confusion in the study of the osmolyte effect.

Addressing the above two aims will lead to a novel and alternative approach to the preferential solvation theory, which provides a clearer physical picture on the roles of water and osmolytes on biomolecular equilibria.

**2. Preferential solvation in the presence of a semi-permeable membrane**

Here we formulate the theory of biomolecular solvation in the presence of a semi-permeable membrane. Let us consider water molecules and solute molecules (denoted by throughout) in a volume enclosed by a semi-permeable membrane, which allows only water molecules to pass through. This local region (denoted by ) enclosed by the membrane is surrounded by the bulk solution (whose volume is ), which consists of water molecules and cosolvent molecules. The entire system, composed of the bulk and the local regions, are under constant pressure () and temperature (). Keeping as a variable for the sake of generality for the moment, the Gibbs-Duhem equations for the exterior (which contains water and osmolyte molecules) and interior (which contains water and solute molecules) can be written in the following manner [6,7,20]:

 (1)

 (2)

where and respectively represent the concentration of the species in the bulk and the local regions, and are the corresponding chemical potentials, and  is the osmotic pressure due to the inaccessibility of the cosolvent into the local region *L* and of the solute to the outside of . (Note that, just as in the classical chemical thermodynamic theories of osmotic pressure, the sole function of the membrane is the selective permeation of molecular species; accordingly, no membrane/surface term appears in the theory). Now we consider the equilibrium conditions. Subtracting Eq. (1) from Eq. (2), and considering the equilibrium condition for water and cosolvent, we obtain

 (3)

Using Eq. (1) to eliminate , Eq. (3) can be rewritten as

 (4)

From Eq. (4) we obtain

 (5)

where

 (6)

is the KB integral, as has been defined in our previous papers and shown to be equivalent to the statistical thermodynamic definition [6,7,20,31]. We will use Eq. (5) later to establish the connection between the KB integrals and volumetric properties derived from the dependence of on . From Eq. (2) we obtain

 (7)

where signifies the volume enclosed by the membrane per solute.

Let us now focus on , the volume enclosed by the membrane. The interpretation of Eq. (7) can be facilitated by the following variable transformation for its l.h.s:

 (8)

Noting that , from the definition of the partial molar volume (in the local region), and also that (as can be shown via Eq. (2)), Eq. (8) becomes

 (9)

Here, signifies the number of water molecules per solute confined within the membrane. Using Eq. (9), Eq. (7) can be rewritten as

 (10)

which can be derived also from the Gibbs-Duhem equation for partial molar volumes in the local region *L*, . Note that Eq. (9) is applicable regardless of the existence and concentration of the osmolytes (species 2) outside the local region, which means it is valid at (zero osmolyte concentration).

**3. Preferential solvation and the osmolyte-dividing membrane.**

We have thus formulated the effect of osmolytes on solute’s chemical potential in the presence of the semi-permeable membrane (Eq. (5)) and on the volume enclosed by the membrane (Eq. (10)). Eq. (10) depends on the number of water per solute, , enclosed within the membrane. Eq. (5) is then dependent on through Eq. (10) as well. At this point, we note that varies with when the membrane volume can be set arbitrarily. According to Eq. (5), this means that the derivative of the free energy of solvation depends on or on (Cf. Eq. (10)). However, the degrees of freedom of a single-phase isothermal system with two solvent species plus a fixed solute, according to the Gibbs phase rule, is two [7]; this is why and are independently determinable for such a system via the KB theory [7]. An additional degree of freedom introduced here should therefore be considered an artefact due to the introduction of a membrane. If the membrane were to remain “hypothetical” or “effective” [3-5], such an additional degree of freedom should be eliminated by fixing *without getting rid of the membrane*. Thus to achieve the two aims set forth in Introduction, we have to impose the following requirement:

**Requirement:** *introducing the semi-permeable membrane (being hypothetical) does not change the thermodynamics of solvation.*

Let us articulate the above requirement:

1. The solvation free energy of a solute (chemical potential of the solute fixed in position), , is not affected by the presence of the semi-permeable membrane (Figure 1).
2. This invariance of is realised by adjusting the volume of the local system enclosed by the semi-permeable membrane, (Figure 1).

Due to the above requirement, we need to consider the free energy of solvation instead of the chemical potential . To do so, we exploit the well-known relationship between the two, i.e., [6,7], where can be interpreted as the chemical potential of a solute whose centre of mass is fixed in position and is the osmotic pressure arising from the freely-moving solute inside the membrane [7]. Using this relationship, Eq. (4) can be rewritten as

 (11)

where is the osmotic pressure due solely to the osmolytes located outside the membrane. To summarise, all the results in Section 2 can be rewritten for fixed solute under the transformation and .

According to Requirement (i), Eq. (5) for a fixed solute should be equivalent to the following relationship in the absence of the membrane [6,7,20]:

 (12)

This is satisfied under the following conditions:

 (13)

 (14)

Thus we have shown that the per-solute volume enclosed by the membrane is . This is identical to the volume confined within the osmolyte-dividing surface introduced by our previous paper [7]. Thus we have shown that the semi-permeable membrane located precisely at the osmolyte-dividing surface can truly make the membrane “hypothetical” [3-5], namely its presence does not affect the thermodynamics of solvation as stated by the Requirements (i) and (ii).

Before concluding this section, note that specifying is equivalent to specifying . Indeed, the combination of Eqs. (10) and (14) yields

 (15)

which shows that is no longer a variable. Requirements (i) and (ii) have thus been fulfilled by identifying the membrane as the osmotic dividing surface [7]; therefore (I) in the introduction has been given a theoretical foundation. Hence the membrane from now onwards will be referred to as the “osmolyte-dividing membrane”.

We have thus clarified how to make the osmolyte-dividing membrane truly “hypothetical” without eliminating its existence, i.e., to be present in the system precisely at the osmolyte-dividing surface in order not to perturb the thermodynamics of solvation.

**4. Preferential solvation as the work of membrane expansion**

 Here we deal with the special case of dilute osmolytes, i.e., . This limit is particularly important in the controversy over OST, i.e., use of osmolytes to estimate biomolecular hydration as discussed in our Introduction [3-5]. At this limit can be expressed as

 (16)

where is the dilute ideal standard chemical potential and is the mole fraction of the species *i*. A small change of (from its value in pure water) that accompanies the introduction of the dilute osmolytes can be expressed as

 (17)

Note that we use from here onwards to denote the infinite dilution limit of osmolytes.

Strictly at this limit, Eq. (17) has a clear physical meaning, as will be demonstrated below. Since van’t Hoff’s law, , holds true at this limit, Eq. (17) can be rewritten as

 (18)

Let us interpret Eq. (18). We have shown that the volume per solute of the osmotic dividing membrane, , is (Eq. (14)), which holds true also at . Using the KB theory, can be interpreted as , which corresponds to Ben-Naim’s pseudo volume (i.e., the excess molar volume of a solute molecule whose centre of mass is fixed in position, which can be related to solute’s partial molar volume and solvent’s isothermal compressibility ) [6,7]. Thus Eq. (18) involves the work of expanding the local region from the initial volume to the final volume done against the osmotic pressure . Note here in particular that the final volume, unlike the initial volume, cannot be expressed as the partial molar volume of the fixed solute in the presence of the cosolvent. Indeed, Eqs. (10) and (14) in combination lead to

 (19)

which contains the contributions not only from the partial molar volume (for the fixed solute) but also from the water molecules confined within the membrane. So what determines this number of confined water molecules? To answer this question, let us exploit (as the solute is fixed and ), through which Eq. (19), which is correct at all cosolvent concentrations, can be rewritten at as

 (20)

where the final approximation is valid for strongly excluded osmolytes, namely [6,7]. Eq. (20) shows that the volume change in Eq. (18) is given by the partial molar volume of water (in the local region) multiplied by , the number of water molecules contained within the osmolyte-excluded volume (i.e., the volume of the osmolyte-dividing membrane). This underscores the importance of water properties in the expansion work provided by Eq. (18). Still, the (effective) interaction between the biomolecule and osmolyte plays a key role in the form of the KB integral , which determines the volume enclosed by the membrane according to Eq. (14). The above interpretation is consistent with our previous work based explicitly on the osmolyte-dividing surface [7].

Thus the work of volume expansion against the osmotic pressure is equivalent to preferential hydration , which endorses the intuitive pictures proposed by Timasheff, Parsegian and others [3-5] ((II) in Introduction) from a rigorous perspective of chemical thermodynamic and the KB theory. Hence it might be necessary to revise the original interpretation of the “free energy of exclusion of osmolytes” from biomolecular surfaces ((III) in Introduction) [3,4]; the work of volume expansion to incorporate more water molecules (Eq. (19)) inside the osmolyte-dividing membrane while keeping the osmolyte excluded would be a more accurate interpretation of Eqs. (18) and (20).

**5. Hydration *versus* osmolyte exclusion.**

As summarised in the Introduction, the most elusive concept in the preferential solvation of biomolecules is the “hypothetical” or “effective” semi-permeable membrane which separates biomolecular vicinity from the bulk solution [3-5], which has been used to demonstrate the equivalence between the depletion and hydration via the use of the semi-permeable membrane [4,5]. Based upon a rigorous chemical thermodynamic consideration, we have defined the “effective” membrane under the condition that introducing such a membrane does not affect the thermodynamics of solvation nor does it introduce an additional degree of freedom against the Gibbs phase rule (Section 3). Hence the volume enclosed by the osmolyte-dividing membrane is no longer a variable, and the position of the membrane was shown to be the identical to the osmotic dividing surface assumed by OST [6] (Section 3).

Based upon this simple setup, we have shown in Section 4 that preferential solvation is equal to the work of volume expansion against the osmotic pressure at the infinite osmolyte dilution limit. We have proposed thereupon that the classical intuitive argument [3,4], that preferential solvation is the “free energy of exclusion” of osmolytes from biomolecular surfaces, should be revised as the work of incorporating more water inside the osmolyte-dividing membrane. Indeed, preferential solvation, namely how biomolecular chemical potential changes upon the addition of the osmolytes, can be expressed as the work (Eq. (18)) of expanding the hydration volume to the osmolyte-exclusion volume . The final volume of the membrane is determined solely by the solute-osmolyte Kirkwood-Buff integral, which is the measure of the volume around the solute from which osmolytes are excluded (depleted), rather than by solute-water interaction. This underscores our previous conclusion that OST actually measures the number of bulk water molecules contained by the osmolyte-dividing surface [6,7], which has been illustrated through the analysis of the experimental data on the effect of osmolytes on ion channel opening, allosteric effects and protein-ligand binding [3-5]. This is in contradiction to (IV) in Introduction.

Thus we have clarified the elusive concept of OST’s “effective” membrane, by clarifying how its bound is set – not in terms of biomolecular hydration but in terms of osmolyte exclusion. We thus support the depletion forces perspective, and have shown that depletion and hydration are not equivalent.

**6. Conclusion**

How cosolvents that are excluded from macromolecular surfaces enhance the folding, binding and self-assembly of macromolecules has been interpreted based upon two different perspectives: depletion forces [3,4,6-8,13,14,17] and hydration forces[2,5,9,10,15]. Whether the two hypotheses are equivalent or contradictory were debated using the “hypothetical” or “effective” semi-permeable membrane [3-5]. However, the ambiguous nature of such a membrane has made the debate inconclusive.

Hence we formulated rigorously the theory of solvation in the presence of a semi-permeable membrane. Such a membrane should not perturb solvation thermodynamics nor should it introduce any additional degree of freedom in order for it to be truly "hypothetical". The KB theory of preferential solvation holds true only when the membrane is positioned at the osmolyte-dividing surface [7]. At the infinite osmolyte dilution limit, we have shown that preferential solvation is equivalent to the work of volume expansion against the osmotic pressure.

We have thus (i) clarified the nature of the osmolyte-dividing membrane and (ii) shown that the preferential solvation theory can be interpreted as the volume expansion work against osmotic pressure at infinite osmolyte dilution limit. The osmolyte-dividing membrane, now rigorously defined, supports the perspective of the depletion force [3,4,6-8,13,14,17], which is not equivalent to the change of hydration.

What has emerged here yet again is the importance of the Gibbs phase rule and the consideration of the degrees of freedom in the interpretation of the osmolyte effect on solvation, which should be dealt with much caution when applying surface and interface concepts to solvation [7].

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**Figure 1:** The basic requirement of our theory. The free energy of solvation of a solute (black) in aqueous osmolyte solutions (**centre**) should remain the same even when the semi-permeable membrane (orange), which only passes through water (blue) but not osmolytes (red), has been introduced. The volume of the solution contained within the membrane () can be specified to fulfil this requirement (**left**). When is different, the requirement cannot be fulfilled (**right**).