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Dehydration of solute-lipid systems: Hydration forces analysis.

Gary Bryant*

Department of Applied Physics, RMIT University, Melbourne (Australia).

Karen L. Koster,

Department of Biology, The University of South Dakota, Vermillion, SD (U.S.A.)

*Corresponding Author:

Tel: 61-3-99252139

Fax: 61-3-99255290

Email: gary.bryant@rmit.edu.au

Abstract

Sorption isotherms were obtained for a range of lipid/sugar/water mixtures. These were analyzed using a simple hydration forces formalism. The results demonstrate that this simple analysis can be used to estimate dehydration parameters for these relatively complex systems. This in turn provides some insight into the location and role of sugars in the hydration behaviour of lipid systems. The relevance of these results to the phase behaviour of lipid/sugar mixtures during dehydration are discussed.

Keywords: Hydration forces, phospholipids, dehydration, sugars, desiccation

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Introduction

It is now well established that the presence of a range of small solutes in desiccation tolerant organisms and organelles plays an important role in their survival during desiccation [1-5], and there has been a great deal of research aimed at establishing the solutes' protective mechanisms. These efforts have largely focused on the ability of a range of solutes to influence the phase transition temperatures of phospholipid membranes, both in the dry state [6-8] and as a function of dehydration (eg Koster et al. [9] and references therein), with a range of experimental techniques being brought to bear (eg [5, 8, 10-12])

At least for simple sugars and a small number of other solutes, the observed effects on membrane transition temperatures at low to moderate hydrations can be modelled and explained semi-quantitatively based on the osmotic and volumetric properties of the solutes and their crystallization and glass forming tendencies [13-18], while at extremely low hydrations direct solute-lipid interactions may play a part (eg [5-7]). While the effects of solutes on phase transitions have received a great deal of attention, there has been relatively little work done on how the presence of solutes affects the osmotic dehydration of the membranes, which is a necessary precursor to the changes in phase transition temperature.

One of the unresolved issues is that of where the solutes reside in a lipid/solute/water mixture at low hydrations. While it has been assumed by most workers that the solutes remain between and near membranes during dehydration, there is now clear evidence that solutes can be excluded from the regions close to membranes (eg [19-21]) and moreover, that the location of the solutes (and therefore their effect on membranes) depends greatly on the history of the samples (eg [9, 15, 22, 23]). This is particularly true at hydrations and temperatures where solutes are near their glass transition, as high viscosities imply very long equilibration times.

In this paper we make a start on trying to understand these issues by presenting water sorption isotherms for a range of lipids in the presence and absence of different solutes. We analyse these data using the hydration forces formalism, as demonstrated by Marsh [24]. Using this formalism we characterize the dehydrative properties of the solute/lipid mixtures, and gain some insight into the osmotic effects of the solutes.

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The relevance of the results to the effects of solutes on membranes during dehydration are discussed.

Materials and Methods

Theory

The phase equilibrium of lipid/water mixtures is extremely complex, with contributions from van der Waals, entropic, and hydration forces. At low to intermediate hydrations the force balance is dominated by the strongly repulsive hydration force (see [25] for a review), which can be written as:

$$P = P_o \exp\left(-\frac{d_w}{\lambda}\right) \qquad (1)$$

where P is pressure (force per unit area), P_o is the extrapolated pressure at zero separation, d_w is the separation between opposing bilayers, and λ is the decay length of the force.

The characterization of the hydration force can be achieved by a number of methods, including the Osmotic Stress Technique (OST) [25], the surface forces apparatus (SFA) [26, 27], and the Freezing Stress Technique (FST) [28].

An indirect but simple method was proposed by Marsh [24] which allows the determination of the hydration parameters for simple systems directly from adsorption isotherms. This method relies on some assumptions regarding the geometry, but has been used successfully to estimate hydration parameters for simple lipid systems. This is done by writing equation 1 in terms of the number of water molecules per lipid n_w, rather than separation:

$$P = P_n \exp\left(-\frac{n_w}{n_\lambda}\right) \qquad (2)$$

where n_λ is the decay length and P_n is the extrapolated zero pressure. In general P_n is not equal to P_o due to changes in lipid area during dehydration (see below).

For a lamellar geometry is it easy to show that d_w and n_w are related by:

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$$n_w v_w = a\frac{d_w}{2} \quad (3)$$

where a is the average area per lipid molecule and v_w is the volume of a water molecule. The factor of 2 arises from the contribution of waters from two lipids in opposing bilayers.

Marsh [24] used a fixed value for a, and in this case $P_n = P_o$ (equations 1 and 2). However, it is well known that the area per lipid decreases during dehydration (see eg [25]), so this must be taken into account. The area may be written [17]:

$$a = a_o\left(1 + \frac{Pd_w}{k_a}\right) \quad (4)$$

where a_o is the area per lipid in the presence of excess water, P and d_w are the force per area and bilayer separation, as before, and k_a is the lateral compressibility. (Note that here we have neglected any effects of changes in area due to temperature, as experimental values of a_o are available at or near the experimental temperatures used here).

So, combining equations 3 and 4 and solving for d_w gives:

$$d_w = -\frac{k_a}{2P}\left(1 - \sqrt{1 + \frac{8n_w v_w P}{k_a a_o}}\right) \quad (5)$$

The pressure P in equation 2 is equal to the experimentally imposed osmotic pressure. Thus by measuring n_w as a function of osmotic pressure, and plotting log P vs. n_w, the P_n and n_λ can be determined (equation 2). If the a_o and k_a are known, then equation 5 can be used to convert n_w to d_w, and then the λ and P_o in equation 1 can also be determined.

The above analysis applies to membrane/water systems. When solutes are present, then the same exponential behaviour can be observed, but now equation 3 must be replaced by:

$$n_w v_w + n_s v_s = a\frac{d_w}{2} \quad (6)$$

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where n_s is the number of solute molecules per lipid, and v_s is the (unhydrated) solute volume. Substituting equation 4 into equation 6 and solving for d_w gives [17]:

$$d_w = -\frac{k_a}{2P}\left(1-\sqrt{1+\frac{8(n_w v_w + n_s v_s)P}{k_a a_o}}\right) \tag{7}$$

If we assume that at low hydration all of the water and solutes are between the membranes (ie none are excluded into a separate phase), then by knowing the number of solutes per lipid in the sample, equation 7 can be used to determine d_w, and hence, via equation 1, the P_o and decay length λ.

Equation 6 assumes that all the solutes and water are contained within the membraneous phase. However, it is possible that some or all of the solutes are excluded, sequestering some water with them. If we assume the most general case, then:

$$\begin{aligned} n_w &= n_w^m + n_w^e \\ n_s &= n_s^m + n_s^e \end{aligned} \tag{8}$$

where the superscripts refer to water and solute in either the membraneous phase (m) or in the excluded phase (e). In this case equation 7 becomes:

$$d_w = -\frac{k_a}{2P}\left(1-\sqrt{1+\frac{8(n_w^m v_w + n_s^m v_s)P}{k_a a_o}}\right) \tag{9}$$

Materials

The phosphatidylcholines SOPC (1,2-distearoylphosphatidylcholine), DLPC (1,2-dilauroylphosphatidylcholine), OPPC (1-oleoyl-2-palmitoylphosphatidylcholine), and DOPC (1,2-dioleoylphosphatidylcholine) were obtained in chloroform from Avanti Polar Lipids (Birmingham, AL, USA), found to be pure using thin-layer chromatography, and used without further purification. The sugars trehalose dihydrate (reduced metal ion content), sucrose (SigmaUltra >99.5% purity), and raffinose pentahydrate (>99% purity) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The sucrose and raffinose were mixed (85% sucrose and 15% raffinose by weight) to correspond with sugars found in desiccation tolerant maize embryos [2].

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Methods

Sugars were mixed with phospholipids in a 1:2 (w/w) lipid-to-sugar ratio to form multilamellar vesicles as previously described [15]. In brief, the chloroform was evaporated under a stream of N_2, followed by resuspenion of the lipid in a solution containing either trehalose or the sucrose/raffinose mixture dissolved in water:methanol (1:1, v/v). After thorough mixing to disperse the lipid and solutes, the samples were dried *in vacuo* at 60°C to remove the methanol. The dry lipid-solute mixtures were resuspended in purified water (Nanopure, Barnstead, Inc., Dubuque, IA, USA) and mixed thoroughly. Control samples containing only the phospholipid were prepared in the same manner. Samples consisting of only sugars were prepared in purified water.

The samples were then loaded into pre-weighed pans and incubated at 28°C above saturated salt solutions that generate known relative vapor pressures [29]. The osmotic pressures (Π) within the samples at equilibrium were calculated using the following equation [30]and assuming that the partial molar volume of water (V_w) does not change at low hydrations:

$$\Pi = -\frac{RT}{V_w}\ln\left(\frac{RH}{100}\right)$$

where RH refers to the percent relative humidity above the saturated salt solutions. The salts used in these experiments, and the osmotic pressures they generate at 28°C [29] are: KNO_3 = 11 MPa, NaCl = 40 MPa, NH_4NO_3 = 65 MPa, $Mg(NO_3)_2$ = 88 MPa, K_2CO_3 = 117 MPa, $MgCl_2$ = 156 MPa, LiCl = 283 MPa, and KOH = 351 MPa. After incubation for one week at the designated relative vapor pressures, the pans were reweighed to determine how much water remained. Dry weights were obtained by drying the samples for at least 16 h at 70°C over P_2O_5 *in vacuo*. Sample hydrations were calculated based on the weights before and after oven drying. The number of water molecules per lipid were determined using the known masses and the molecular weights of the components.

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Results and Discussion

Figure 1 shows hydration isotherms for four lipids: (a) DLPC, (b) DOPC, (c) OPPC and (d) SOPC. Each plot shows experimental points for the pure lipid, the lipid with an 85:15 sucrose/raffinose mixture (lipid/sugar 1:2 w/w), and the lipid with trehalose (lipid/sugar 1:2 w/w). The vertical axis is the natural log of the osmotic pressure (in MPa), and the horizontal axis is the number of water molecules per lipid molecule remaining in the sample at that osmotic pressure.

For the pure lipids, the plots are straight lines over this range, allowing fits using equation 2 to give P_n and n_λ. Other phosphatidylcholines studied gave similar results (data not shown). The parameters from the fits to the straight line regions of the data are given in Table 1. The results for both P_n and n_λ are in broad agreement with the analysis of Marsh [24], though as the data analysed by Marsh were from a range of sources and carried out at a range of temperatures, there is some variation.

In the presence of sugars, the data appear linear at low water contents, but deviate from linear at large water contents, when van der Waals and fluctuation forces are expected to play a significant role (e.g. [31]). The lines represent fits to the linear part of the data, and the fit parameters are also given in Table 1.

Looking at the parameters in Table 1, a few points become apparent. First, there is very little variation in the P_n values for all the pure lipids. As they all have the same phosphatidylcholine headgroup, this is not surprising. Second, the presence of the sugars does not significantly change the P_n values, though they are all slightly higher.

More interesting is the value of the decay parameter n_λ. For pure lipids $n_\lambda \sim 2$ (within the relatively large errors) for all lipids. In the presence of the solutes, n_λ rises to somewhere between 6 and 8.

Of course the n_λ here represents the total amount of water in the sample and does not necessarily indicate that the distance between bilayers is increased – it could equally well be explained by some or all of the sugars being present in a separate excluded phase.

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In order to complete the interpretation of the data shown here, we need a better understanding of where the solute resides in lipid-solute mixtures at low hydration. At high hydration, with an excess water phase, it has been shown that the concentration of solutes between the lamellae is about 1/3 less than the concentration in the excess water phase [21]. Similar evidence of exclusion was observed using the Surface Forces Apparatus in excess water [20].

However, there are several lines of evidence to suggest that there is no significant phase separation for the systems studied here. First, we know that a large amount of solute is incorporated into the membranes, because of the dramatic reduction in membrane phase transition temperature in the presence of solutes, which is not seen in the presence of solutes large enough to be fully excluded [9]. Second, if there were a separate sugar/water phase, then at the low hydrations studied here, some of the solutes would likely crystallize (as may be observed for pure sugar/water mixtures at these water contents). Crystallization would be seen in Differential Scanning Calorimetry (DSC) scans if the sample is scanned to temperatures above the crystalline melting point, and could be inferred from anomalous water contents. Indeed for some samples such effects have been observed [32], but any samples showing evidence of crystallization have been excluded from the present analysis. Third, the glass transition of the solutes in the presence of lipids is found to be either equal to, or lower than in the pure state at the same osmotic pressure [9, 15]. This is the reverse of what would be expected following the measurements of Demé and coworkers [21]. These measurements showed that where there was a separate water phase, the sugar concentration outside the lipid would be higher than inside. Thus if there were an excluded phase with a significant amount of sugar, it should have a higher concentration, and thus a higher glass transition temperature than would be observed for the solute entrapped between bilayers.

For the samples studied here there is no evidence of such phase separation (i.e., only a single glass melt was detected using DSC ([15] data not shown here), however, such effects are likely to be a function of sugar concentration. Shalaev and Steponkus [33] found for DOPE/sucrose mixtures that phase separations occurred at low hydration for a molar ratio of lipid:sucrose of 1:2, but not for molar ratios of 1:1 or 2:1. The

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results reported in the present work are at a lipid:sugar ratio of 1:2, yet no evidence of phase separation was seen. The difference is likely to be due to the fact that phosphatidylethanolamines (as used by Shalaev and Steponkus) hydrate less strongly than phosphatidylcholines (as used here). Also the fact that PEs form inverted hexagonal phases may increase the propensity for phase separation from sugars.

Our results suggest that in a well mixed sugar/lipid/water sample at low hydration, most if not all of the sugars will exist between the bilayers. However, this does not necessarily imply that the solutes are uniformly distributed within the interbilayer space. Experiments using the Freezing Stress Technique (FST) [19] have found evidence that solutes such as trehalose are partially excluded from the region closest to the membrane. These results are consistent with the suggestion [21, 34] that the first hydration layer of the membrane is not available for the solute. In other words, the solutes are concentrated in the middle of the interbilayer region.

How would the distribution of solutes between membranes affect the sorption isotherms? The two extreme cases are that the solutes are excluded from the first hydration layer of the lipids – in this case one would expect that the total hydration of the lipid/sugar mixture at a particular osmotic pressure would be simply the sum of the hydrations of the pure lipid and sugar – i.e. there is no water sharing. If, however, the sugars are predominantly interacting with the lipid interface, one would expect the total hydration of the mixture to be less than a simple sum of the components.

Figure 2 shows a log-log plot of the number of waters per solute molecule (n_w/n_s) vs. the osmotic pressure for sucrose, trehalose, and sucrose/raffinose mixtures (open symbols). Data for sucrose from other sources are also included on the graph [19, 35]. For osmotic pressures less than about 10 MPa, the behaviour is linear. Above 10 MPa, however, the data dips below the straight line fit. A separate straight line fit has been made to the data above 10 MPa. Note that at high osmotic pressures (greater than about 10 MPa), these experiments are plagued with problems of non-equilibration and crystallization [32]. The data shown in Figure 2 are for samples which showed no evidence of crystallization, and where the masses had stabilized. The excellent agreement with other methods at intermediate osmotic pressures lends extra support to the validity of these measurements.

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From the fit, we can determine the amount of water that pure sugar will adsorb as a function of osmotic pressure. Figure 1 provides similar data for pure lipids. Using these data we can compare the experimental sorption isotherms for the mixed systems with what would be expected based on a linear sum of sugar and lipid hydrations. This is shown in Figure 3 for each of the lipids with sucrose/raffinose (trehalose results are similar). The dotted line is the fit for the pure lipid, the dashed line is the fit for the pure sugar, while the solid line is this value multiplied by the number of solutes per lipid in these samples. The bold line is the sum of these contributions. The experimental hydrations are the filled circles. The graph shown uses the fit at high pressures (Figure 2), but using the low pressure fit does not significantly alter the results.

The data here show total additivity of the waters of hydration. This implies either that the water (and therefore at least some solutes) is either in a completely separate phase from the lipids (which has been discounted, and in any case is highly unlikely) or that the lipids and solutes are locally separate. These results are by no means conclusive; however, given the results of the other techniques, the experimental hydrations are consistent with the idea that there is some partial exclusion of the solutes from the first hydration layer. Similar behaviour is seen for the other three lipids studied, and for all four lipids with trehalose instead of sucrose/raffinose. Note that Shalaev and Steponkus [33] also found total additivity of hydration in DOPE/sucrose samples where there was no phase separation, and saw lower than predicted hydrations for samples where phase separation was observed.

Having established that the bulk of solutes are incorporated into the membrane phase, we can use equation 7 to calculate the average separation between the membranes d_w. A graph of ln (P) vs. d_w for DLPC is shown in Figure 4. As would be expected, the presence of the sugars increases the average distance between the bilayers, though the relationship remains linear. Table 2 shows the results of the fits to the linear part of the curves.

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The results are self-consistent, within the difficulties associated with the determination of hydration forces parameters (for a discussion see [25]). (Note that the location of the solutes within the interbilayer space does not affect this model)

As far as we are aware, the only measurements of the hydration force of lipids in the presence of sugars are those of [19]. However, the Nuclear Magnetic Resonance (NMR) technique they used provides quantitative data only up to about 20 MPa.

We have chosen to use 145 mN/m for DLPC, and 200 mN/m for the longer chain lipids. The exact values only affect the fit parameters in a small way, and does not affect the linearity of the data. Given the uncertainties in these parameters, the fit parameters are accurate to only about 20%.

This simple analysis of lipid-sugar-water mixtures shows that a range of information can be obtained from this relatively simple experiment. In the case of these relatively simple systems, other techniques, such as Small angle X-ray scattering (SAXS), Neutron scattering or NMR can be used to obtain quantitative information about the structural parameters, and thus more accurately and unambiguously determine the hydration force parameters. This work is currently underway.

However, the disadvantage of such techniques is that they cannot be easily applied to more complex systems, such as macromolecules that have complex geometries (e.g. proteins), cells and tissues. The value of the analysis developed here is that it may, in principle, be applied to these more complex systems, and such analyses are currently underway.

Conclusions

We have demonstrated that a simple hydration forces analysis of adsorption isotherms for lipid-sugar-water mixtures can be used to obtain hydration force parameters. This in turn provides some insight into the location and role of sugars in the hydration behaviour of such systems, which in turn affects the phase behaviour. Similar studies on more complex systems may yield insights which cannot be achieved by more direct experimental methods.

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Table 1

	Solute	P_n (MPa)	n_λ
DLPC	--	381	2.4
	S/R (1:2)	402	6.4
	Tre (1:2)	423	6.8
DOPC	--	340	1.7
	S/R (1:2)	366	7.4
	Tre (1:2)	362	8.5
OPPC	--	394	2.0
	S/R (1:2)	396	7.4
	Tre (1:2)	416	7.4
SOPC	--	411	2.5
	S/R (1:2)	511	8.1
	Tre (1:2)	640	7.1

Table 1: fit parameters for the lines shown in Figure 1. All fits are to the low hydration linear part of the curve (consisting of 4-6 data points). The errors calculated from the fits are 5% or less for the P_n values, and 10-20% for the n_λ values.

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Table 2

	Solute	P_o	λ	R&P	R&P λ
		MPa		MPa	
DLPC	--	388	.18	400	0.20
$a_o = 0.640\ nm^2$	S/R (1:2)	1414	.99	--	--
$k_a = 145$ mN/m	Tre (1:2)	1377	.97	--	--
DOPC	--	320	0.15	400	0.21
$a_o = 0.721\ nm^2$	S/R (1:2)	1344	1.21	--	--
$k_a = 200$ mN/m	Tre (1:2)	1286	1.19	--	--
OPPC	--	365	.17	--	--
$a_o = 0.701\ nm^2$	S/R (1:2)	1674	1.10	--	--
$k_a = 200$ mN/m	Tre (1:2)	1483	1.13	--	--
SOPC	--	410	0.21	300	0.20
$a_o = 0.643\ nm^2$	S/R (1:2)	990	1.55	--	--
$k_a = 200$ mN/m	Tre (1:2)	980	1.50	--	--

Table 2: shows the fits to the data shown in figure 1, assuming that all the solute and water is in the membrane phase, and applying equation 7. Also shown for comparison are values from Rand and Parsegian [25] for three of the pure lipids. The parameters used in the calculations are from the literature: a_o values are from [25], except OPPC, which is taken as the average of the areas of DPPC and DOPC in the fluid phase; k_a values are scarce - [36] report $k_a = 200 \pm 13$ mN/m for SOPC and $k_a = 145 \pm 11$ mN/m for DMPC, both in the fluid phase, and 855 ± 140 mN/m for DMPC in the gel phase.

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Figure Captions

Figure 1. Log of osmotic pressure vs. number of water molecules per lipid for (a) DLPC, (b) DOPC, (c) OPPC and (d) SOPC. Each frame shows data and fits for pure lipid (diamonds, bold line), lipid plus sucrose/raffinose (squares, thin line) and trehalose (pluses, dashed line).

Figure 2. Figure 2 shows a log-log plot of the number of waters per solute molecule (n_w/n_s) vs. the osmotic pressure for sucrose, trehalose, and sucrose/raffinose mixtures (open symbols). Data for sucrose from other sources are also included on the graph [19, 35].

Figure 3. Number of water molecules per lipid in lipid/sucrose/raffinose mixtures for (a) DLPC, (b) DOPC, (c) OPPC and (d) SOPC. The dotted line is the fit for the pure lipid (from figure 1), the dashed line is the fit for the pure sugar (from figure 2), while the solid line is this value multiplied by the number of solutes per lipid in these samples. The bold line is the sum of these contributions. The experimental hydrations are the filled circles. The graph shown uses the fit at high pressures (figure 2), but using the low pressure fit does not significantly alter the results.

Figure 4. Log of osmotic pressure vs. estimated interlamellar separation d_w for (a) DLPC, (b) DOPC, (c) OPPC and (d) SOPC. Each frame shows data and fits for pure lipid (diamonds, bold line), lipid plus sucrose/raffinose (squares, thin line) and trehalose (pluses, dashed line).

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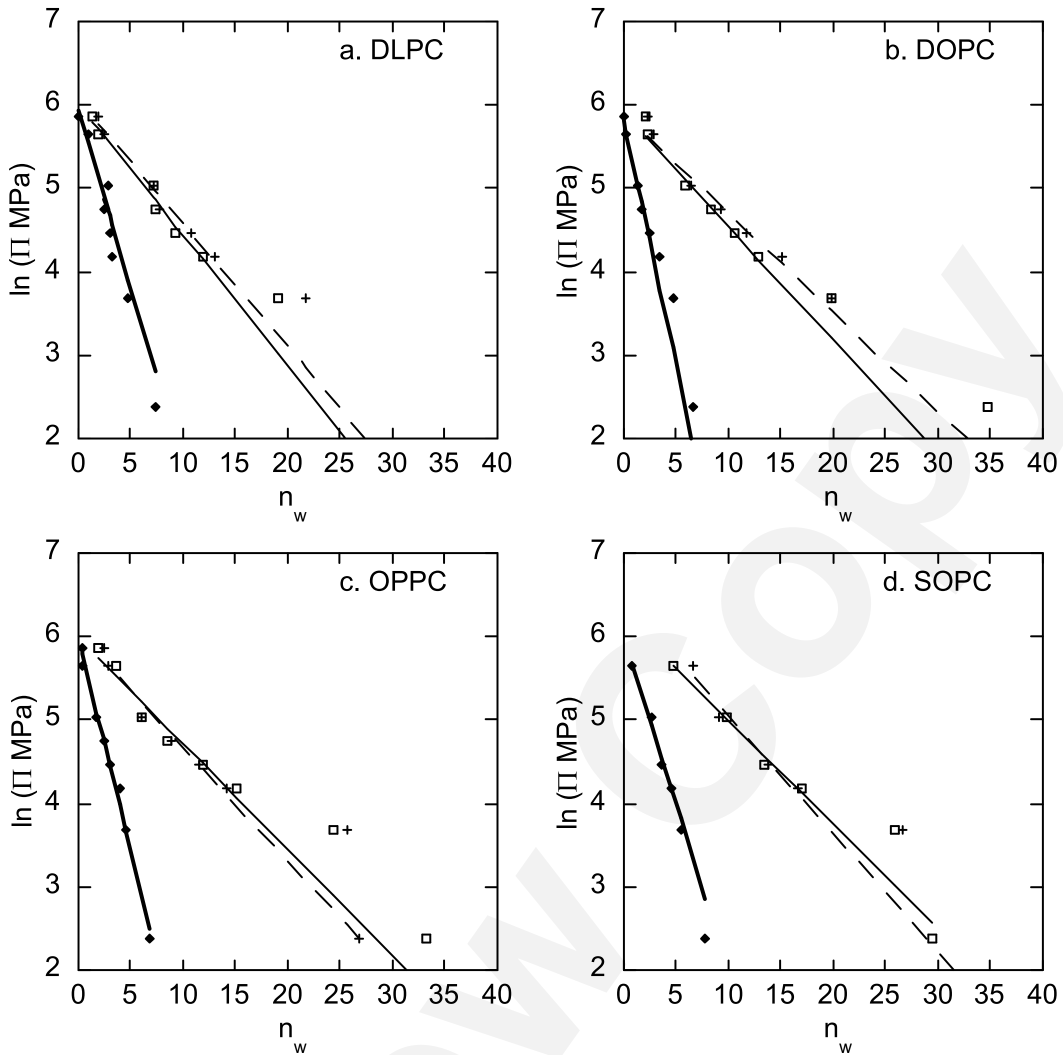

Figure 1

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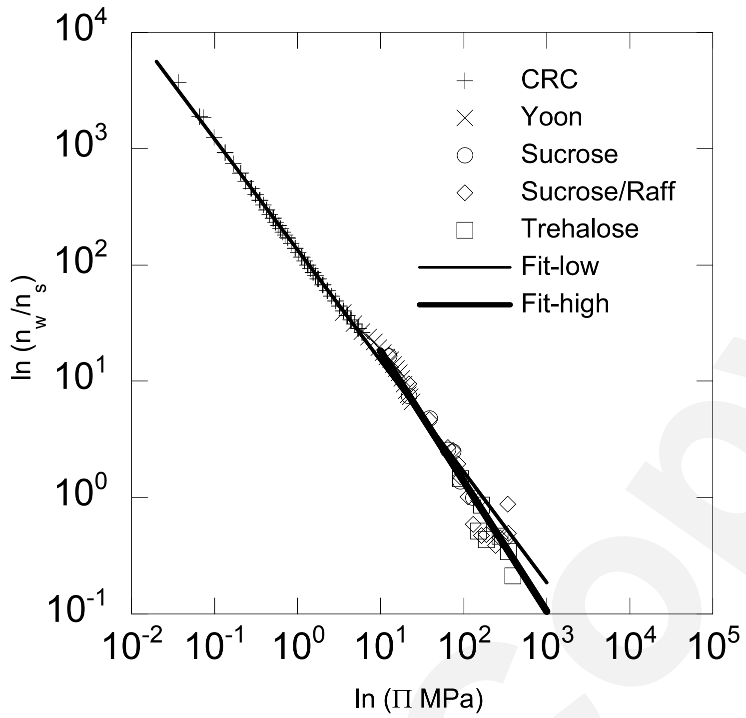

Figure 2

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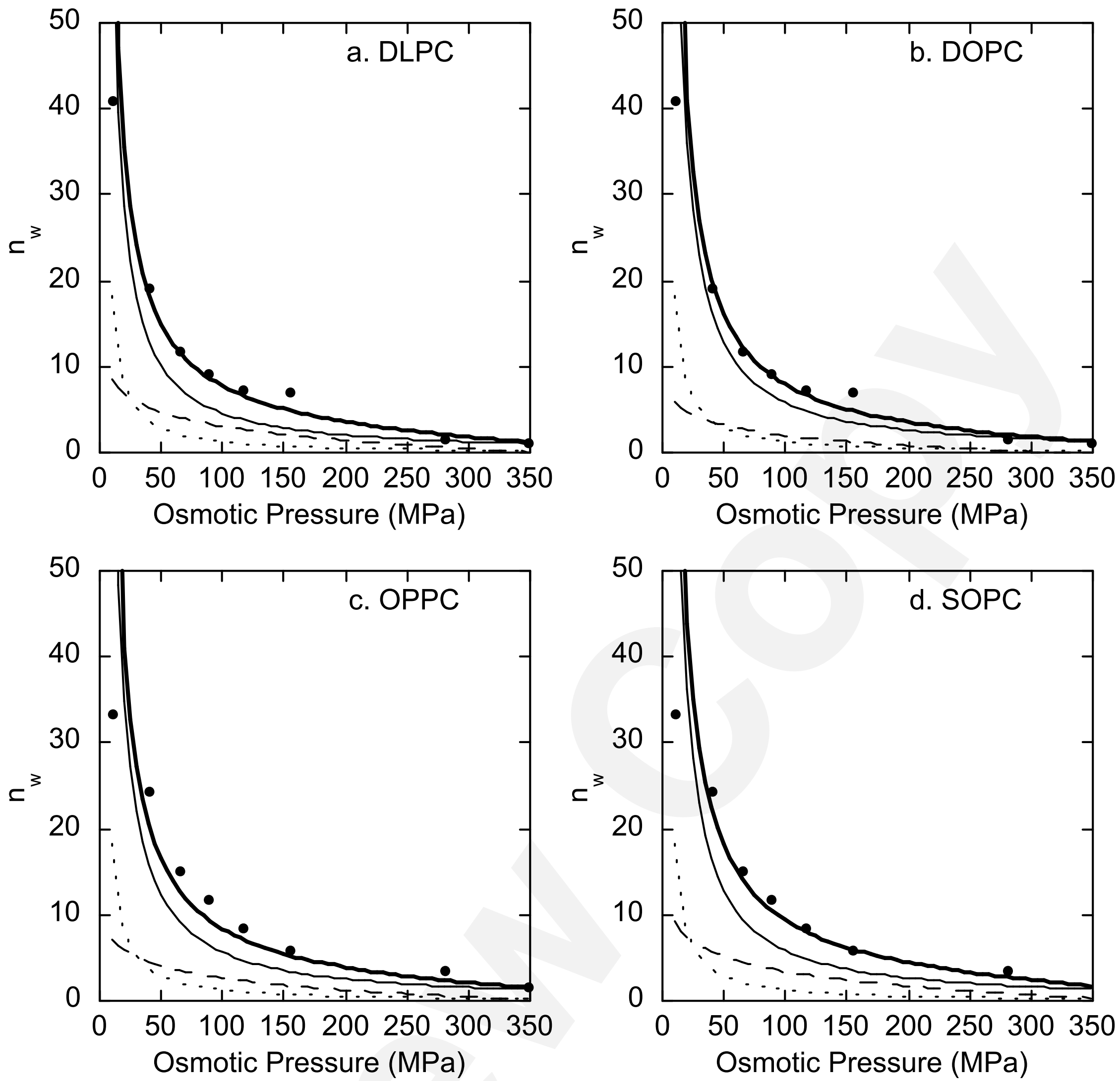

Figure 3

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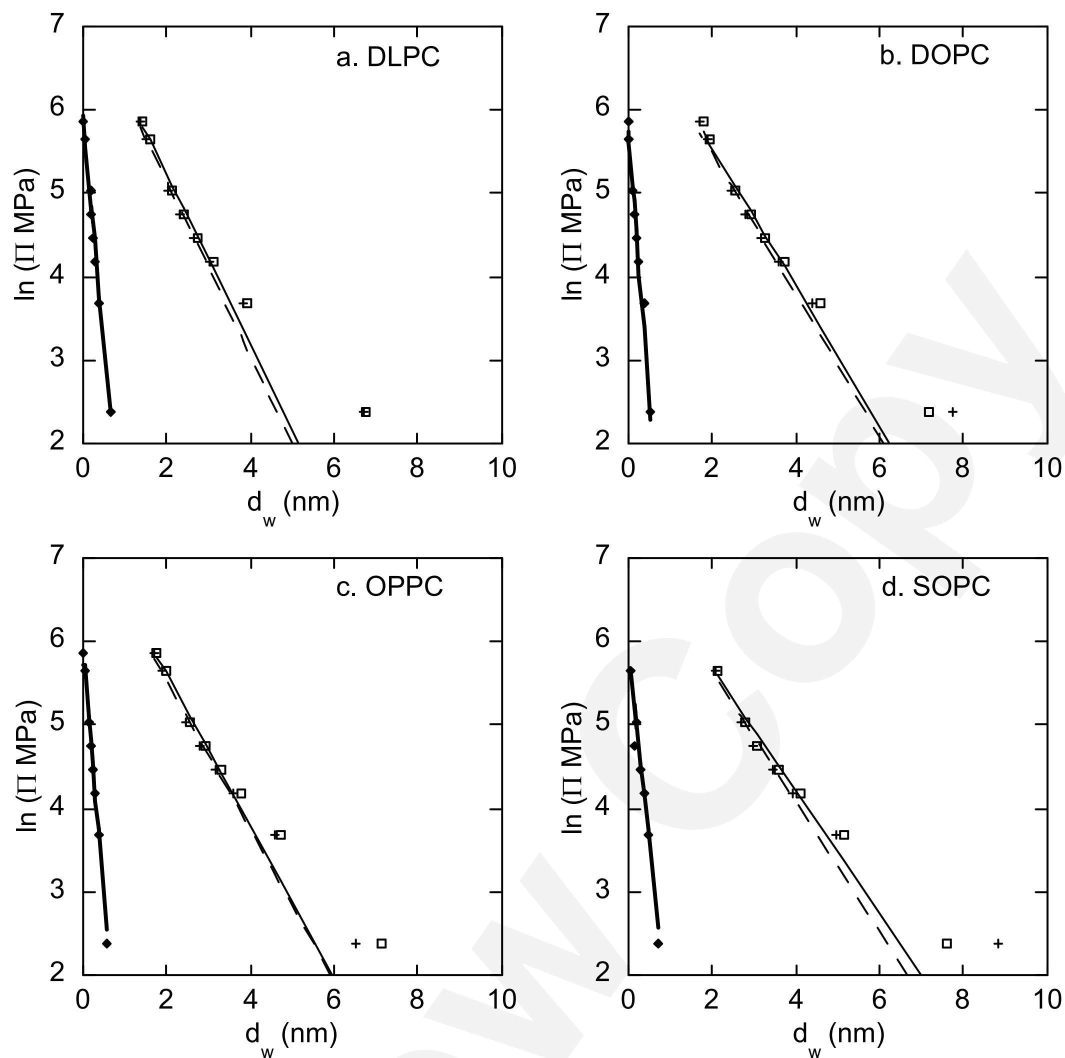

Figure 4

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