Measurement of sugar depletion from uncharged lamellar phases by SANS contrast variation

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We applied small-angle neutron scattering (SANS) contrast variation to samples where a microphase separation occurred. The samples contain multilamellar vesicles in equilibrium with excess "solvent" that produce a very common powder pattern in SANS: a Debye-Sherrer ring produced by the regular bilayer packing superposed to a sharply decaying Porod behaviour. These two features of the SANS pattern have distinct contrast match points (CMP). We exploit here the small angle signal to determine the partition of sugars between two coexisting microphases. The net result is an exclusion of small sugar molecules from the liquid crystalline domains of the sample. We discuss this exclusion in relation with the observed maximum swelling, headgroup hydration and bilayer softening induced by the presence of the sugar molecules.

1. Introduction

Microphase separation is a very common situation in colloidal solutions. For example, the phase equilibria of anisometric colloids (Langmuir, 1938) results in the coexistence of ordered and diluted domains in colloidal solutions. The formation of "tactoids" (Kruyt, 1952), is described as the most common situation where small amounts of a birefringent phase are in equilibrium with a sol. This type of samples have often the appearance of clear gels which are very difficult to separate in two "pure" phases. The equilibrium phase diagram can only be determined by chemical analysis of well separated phases. In some cases, for example when large polyelectrolytes coexist with concentrated clay dispersions, the complete separation of the two phases allows the determination of the osmotic pressure, *i.e.* the thermodynamic underlying the coexistence of the two distinct microphases (Morvan *et al*., 1994).

A very important case is the analysis of lamellar phases used as model membranes, such as the classical zwitterionic DMPC/water dispersions. The binary phase diagram of this system is well known (Janiak, Small & Shipley, 1976; Smith *et al*., 1988) : at room temperature, the DMPC lamellar liquid crystal is in equilibrium with excess water at the so-called maximum swelling (D^* = 60.4 Å). At that periodicity, the water content is 41 % corresponding to 24.9 Å thick water layers separating the membranes in L_α domains. An ubiquitous founding paper of biological membrane physics (LeNeveu *et al*., 1977) has proposed a quantitative explanation of the observed maximum swelling, using an original experimental procedure. The *ansatz* is that at the maximum swelling, the difference between the osmotic pressures of the coexisting phases is zero. Now, if one of the phases is pure or almost pure water (lipid at the CMC), then the total osmotic pressure in the concentrated phase is zero as well, resulting from the balance between dispersion forces and short range "hydration" repulsions, both independently measurable (Lis *et al*., 1982). The critical test of this basic hypothesis is the measurement of the maximum swelling *versus* sugar content using different sugar concentrations. It has been inferred (LeNeveu *et al*., 1977) that the main effect of sugar addition was to shield the dispersion force by matching the permittivity, thus producing a minimum in the attractive dispersion force (Parsegian & Weiss, 1981). The associated maximum swelling observed when sugar is added to zwitterionic lecithin/water dispersion has been explicitly calculated by Parsegian and has been shown to be consistent with the experimental observation.

However, the identification of the dominant interactions in biphasic samples involving a lamellar phase in coexistence with a "solvent" as a reservoir requires as a first step the knowledge of the exact content of each of the two microphases in equilibrium. When macroscopic separation is possible, the easiest way is a direct dosage of the third component together with a measure of the osmotic pressure. For example, cationic bilayers in the presence of excess salt induce a strong Donnan exclusion mechanism which explains quantitatively the observed stability limit *versus* dilution of the DDAB/water/salt system (Dubois *et al*., 1992). In the case of added polymers or complex sugars (Demé, 1995), macroscopic phase separation using centrifugation is not reliable since ultracentrifugation may separate self-assembled aggregates instead of the microphases.

The aim of the experimental method described here is to demonstrate that contrast variation can be used to dose the content of the two coexisting phases without requiring the delicate step of macroscopic separation. The ternary system used is DMPC in the presence of an excess "solvent". The solvent is a concentrated sugar solution, similar to those used a long time ago to increase contrast in small-angle x-ray scattering (SAXS) experiments. The ternary sample is a microphase separated biphasic sample, with a lamellar phase at "maximum swelling" in equilibrium with excess solvent. This lamellar phase appears in the form of multilayer vesicles designed as onions or MLVs (Multilamellar Vesicles) producing Maltese crosses under polarising microscope. These are formed on a mesoscopic scale, *i.e.* they are too small for easy separation from the pure coexisting solvent, but however large enough to produce sharp Bragg peaks whose width is not limited by the number of layers (finite crystal case), but by the interlayer fluctuation (Dubois & Zemb, 1991). The aim of the SANS contrast variation experiment is to determine directly the sugar content of the "excess solvent" and the sugar content of the water forming the multilayer vesicles suspended in the excess solvent. *A priori* these two concentrations cannot be considered safely as equal, and the ratio of the two concentrations cannot be estimated from any current solvation predictive theory as in the case of salt in charged phases (Dubois *et al*., 1992).

2. Materials and methods

We used two sugars, glucose and fructose as model host molecules to investigate the swelling behaviour of the L_α domains, and partially deuterated sugars: 2D-fructose, 2D-glucose and 7D-glucose for the contrast variation experiment.

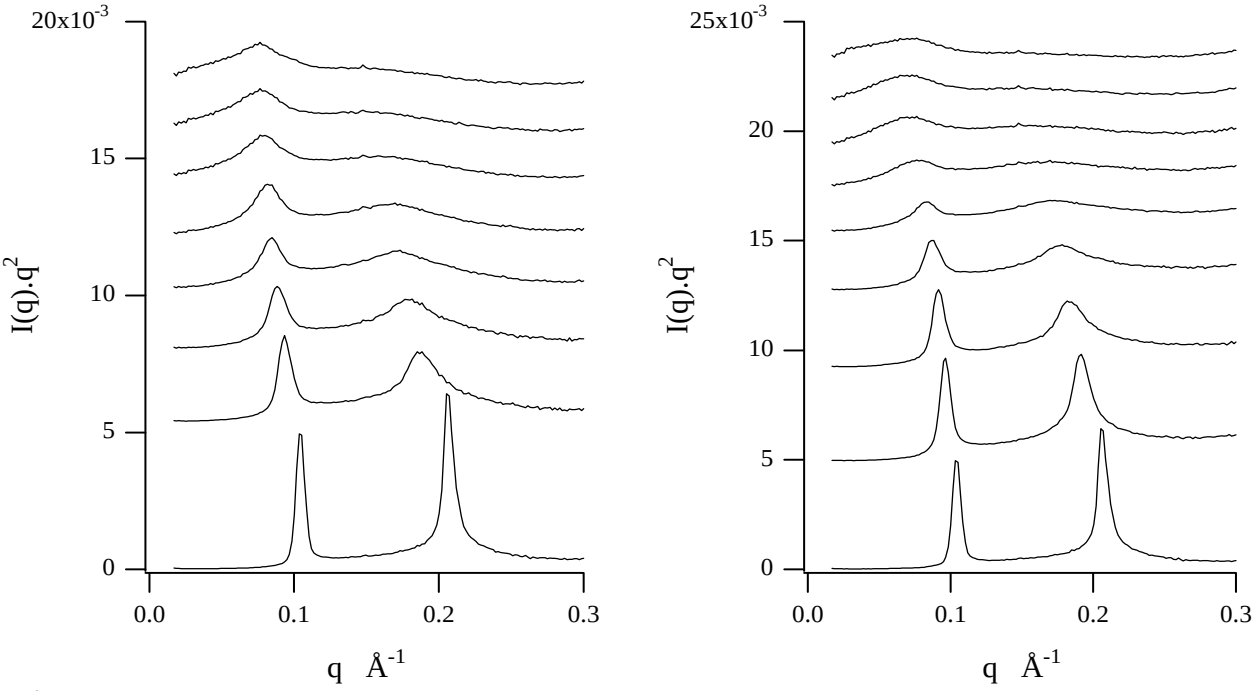

Figure 1
SAXS produced by DMPC suspensions prepared with increasing concentrations of glucose (left) and fructose (right). The volume fraction of the lipid (Φ_L in the text) is kept constant to 0.20 and that of the "solvent" ($\Phi_W + \Phi_S$) is 0.80 for all samples. From $\Phi_S = 0$ (lower curve) to 0.45 (upper curve).

Glucose and fructose were obtained from Fluka (Buchs, Switzerland) and deuterated sugars were from Eurisotop (Saclay, France). DMPC was obtained from Avanti Polar Lipids (Alabaster, AL)

SANS experiments were performed on D11 at the High-Flux-Reactor of the Institut Laue-Langevin. All collected 2D-data were isotropic as expected for random dispersions of spherical lamellar aggregates. They were radially averaged and normalised to monitor and transmissions, and corrected for sample container scattering, background scattering of the instrument and detector efficiency. Finally, a flat background due to incoherent scattering produced by the sample was subtracted to the intensity. Since all investigated samples were prepared in the biphasic region of the binary DMPC-water phase diagram, we took care that no macroscopic phase separation occurred, so that the scattering was produced by exactly the same amount of material for all contrasts. This was an important requirement for the contrast variation experiment to be successful.

SAXS patterns have been recorded on a home-build laboratory X-ray camera in pinhole geometry using CuKα radiation associated to a two dimensional detector. Modifying the original Huxley-Holmes design by separating the mirror and the focussing monochromator reduces the parasitic background and allows routine measurement of weak signals, such as the second damped Bragg peak produced by stacks of soft bilayers measured here with counting times of typically one hour on a laboratory X-ray source (Zemb *et al*., 1999).

Sample preparation: An extremely important requirement in sample preparation is the homogeneity of the samples, in terms of mixing the "solvent" with the lipid. This is a very critical step, particularly when the "solvent" is a polymer solution. In a previous work (Demé *et al*., 1996), we have shown that an immediate shortcut towards concentration equilibrium is to use freeze-drying of an extremely dilute solution (typically less than 1%), followed by careful rehydration of the sample by the required amount of water. We have checked that this procedure allows to obtain immediately the same spacing as after several weeks of equilibration times, when mixing of the sample is completed by molecular diffusion (Demé *et al*., 1997).

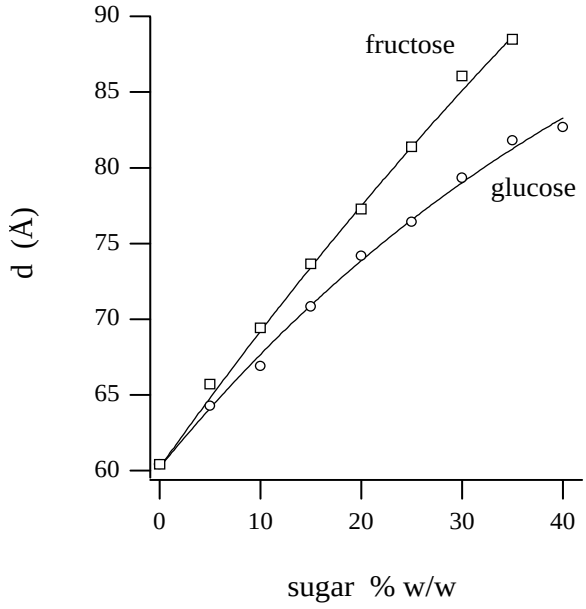

Figure 2
Maximum swelling of the DMPC lamellar phase observed at equilibrium with concentrated sugar solutions.

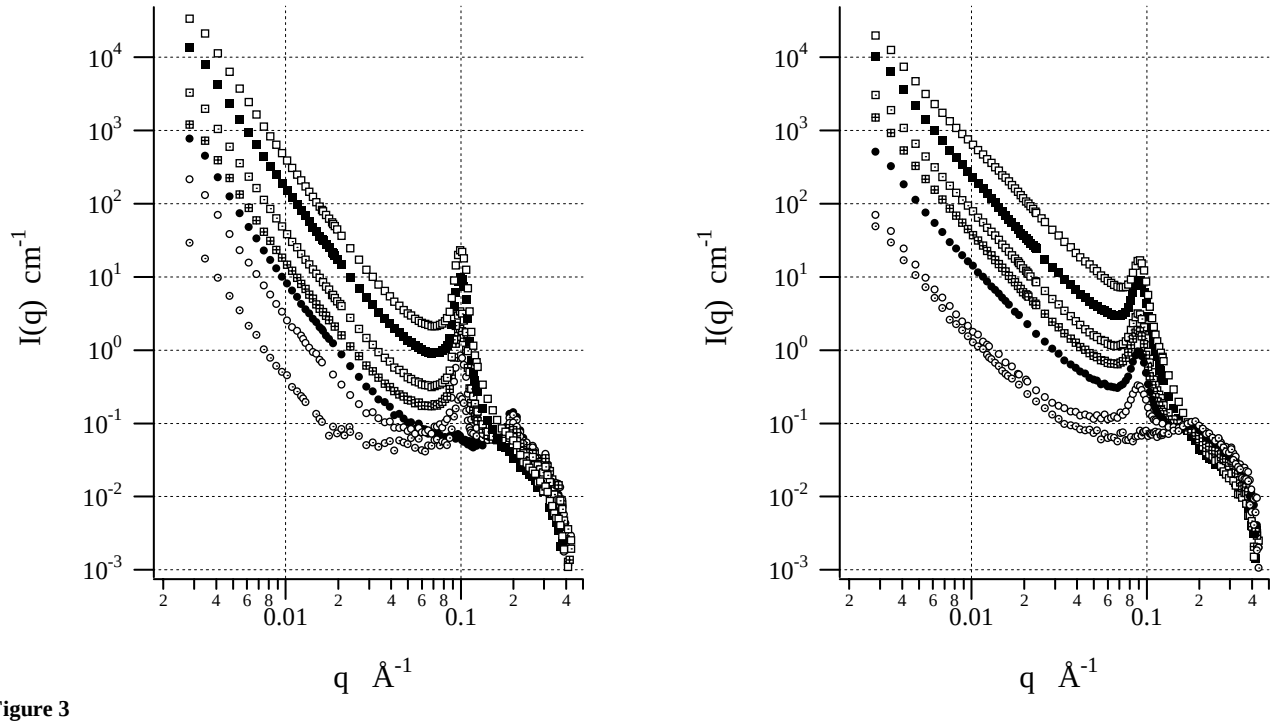

Figure 3
SANS produced by pure DMPC (left) and DMPC + 2D-glucose at 11.5% (right) for increasing water contrast (from bottom to top). The lamellar phase is at maximum swelling observed with water or the concentrated sugar solution in equilibrium.

3. Results

3.1.Coexistence of vesicles with excess solvent

Figure 1 shows SAXS patterns with increasing concentration of added glucose (left) and fructose (right) as a function of the length of the scattering vector $q=4\pi \sin(\theta/2)/\lambda$, where θ is the scattering angle and λ is the wavelength. The vertical scale is in $I(q)q^2$ units in order to get rid of the q^{-2} dependence due to the flat bilayers. In both cases, the monotonic swelling and the simultaneous softening is evidenced by the progressive broadening of the Bragg reflections combined with a shift of the peaks towards low q. Since the 1, 2 indexing is kept for any sugar content explored in the range from 0 to 45% in the solvent, the interlamellar spacing is always increasing with the sugar content, without the presence of the maximum at 20% as previously described (LeNeveu *et al*., 1977). The periodicities associated to the samples are shown on Fig. 2, which emphasises that the global effect of addition of a small hydrosoluble molecule such as glucose or fructose favours repulsive interactions and do not induce liquid crystal shrinking due to depletion mechanisms such as those induced by hydrosoluble polymers. In order to discuss the result obtained here, *i.e.* a monotonic swelling associated to bilayer softening, we turn now to the composition of the coexisting phases, as allowed by SANS contrast variation.

3.2. Determination of contrast match points

The SANS patterns obtained for different D_2O/H_2O ratios are shown on absolute scale on Fig. 3. The double logarithmic scale emphasises the separation in reciprocal space of the sharply decaying signal produced at low angles from the Bragg peak corresponding to the periodicity of the stack close to 60 Å for solvent. On average over micronic distances, the vesicles and the excess solvent have not the same scattering length density, thus producing the long range fluctuation. The apparently smooth Porod-type decay is produced by the total interface between lamellar domains and the excess sugar solution. Because of the

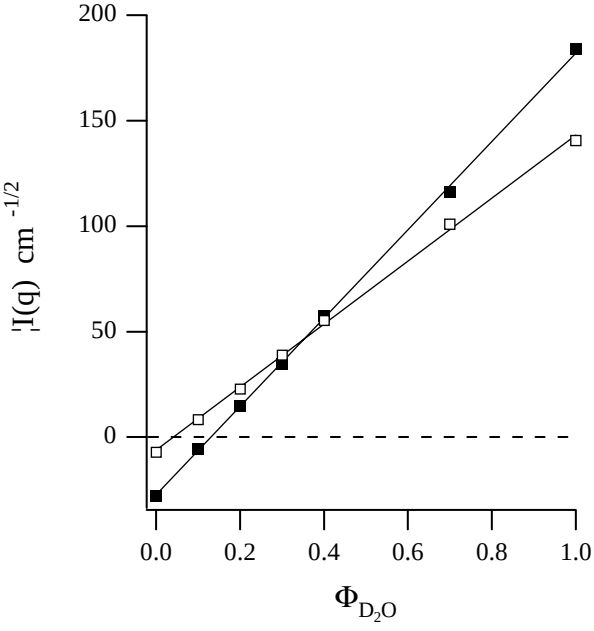

Figure 4
Comparison of the onion-excess solution CMP obtained with pure DMPC (■) and in the presence of 2D-glucose (□). The linear fit to the data yields 0.34 x 10^{-6} Å^{-2} for the pure lipid and -0.25 x 10^{-6} Å^{-2} with 2D-glucose.

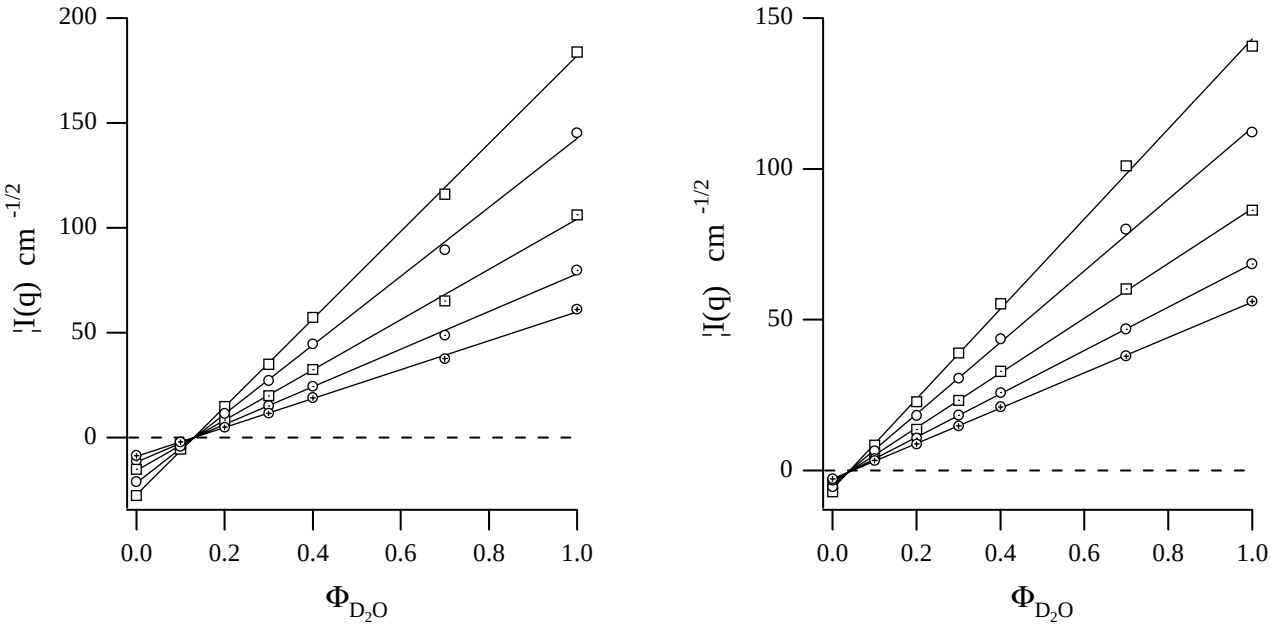

Figure 5
Plots showing the q-independent determination of contrast match points at low angles. Left: pure lipid. Right: lipid + deuterated sugar. Each curve corresponds to a given finite q value whose points are taken from the different contrasts measured. From 2.8 x 10^{-3} Å^{-1} (upper curve) to 5.5 x 10^{-3} Å^{-1} (lower curve).

high polydispersity of the lamellar aggregates, the oscillations of the form factor disappear, leading to the q^{-4} decay. The basic observation is that the zero contrast points observed with the pure lipid and in the presence of 2d-glucose differ significantly (Fig. 4): our aim is to deduce the concentration of sugar molecules from exploitation of these two separated CMPs.

We first have to verify that the CMP is not depending on the *q*-value considered to draw the classical $\sqrt{I}$ *vs*. Φ_{D2O} plot. Figure 5 shows that whatever the q-value considered, the match point for the strong decay at low-*q* is always observed for Φ_{D2O}= 0.131 for the pure lipid (left) and Φ_{D2O}= 0.045 in the presence of the sugar (right). This corresponds to a density ρ_w of the mixture matching the multilamellar vesicles of 0.34 x 10^{-6} Å^{-2} and -0.25 x 10^{-6} Å^{-2}, respectively.

3.3. Determination of sugar volume fraction in the solvent and inside the onions.

We now turn to the quantitative evaluation of the sugar (solute molecule), using three experimentally measured quantities. We note Φ_X the global volume fractions of component *X* and ψ_X the volume fractions of component *X* in the respective microphases. From the known composition of the sample, we have the following global volume fractions of the compounds:

a) in pure water:

$\Phi_L = 0.200$
$\Phi_W = 0.800$

b) with sugar

$\Phi_L = 0.200$
$\Phi_W = 0.708$
$\Phi_S = 0.092$

From the periodicity of the lamellar stack in the onions, we can deduce $\Psi_L = 2t/D^* = 0.509$, using the known bilayer thickness in excess solvent $2t$ = 35.5 Å (Janiak *et al*., 1976), and the value of D^* = 69.8 Å. From this, it follows that $\Psi_S + \Psi_W$ = 0.491.

Deduction of sugar partition between the two microphases proceeds as follows:

a) the measured contrast match point in absence of sugar gives the mean scattering length density of the lipid $\langle\rho_L\rangle$:

$$\langle\rho_L\rangle = 0.34 \times 10^{-6}\ \text{Å}^{-2}$$

b) the measured contrast match point in the presence of deuterated sugar gives the scattering length density of the water required to match the membrane in the presence of added deuterated sugar:

$$\rho_W = -0.25 \times 10^{-6}\ \text{Å}^{-2}$$

c) finally, at small angles, the relation to be resolved at the CMP is:

$$\langle\rho_{onion}\rangle = \rho_{Solution}$$

where the scattering length densities of the coexisting phases can be written as follows:

$$\langle\rho_L\rangle\Psi_L + \rho_S\Psi_S + \rho_W\Psi_W = \rho_S\Psi_S' + \rho_W\Psi_W'$$

with

$$\Psi_L + \Psi_S + \Psi_W = 1$$

The primes relate to volume fractions in the excess "solvent".

The scattering length density of the sugar, corrected for hydrogen exchange with water at $\Phi_{D2O} = 0.045$ is $\rho_S = 2.78 \times 10^{-6}$ Å^{-2} and that of the lipid is known from the CMP in absence of sugar: $\langle\rho_L\rangle = 0.34 \times 10^{-6}$ Å^{-2}. The values of volume fractions forming the "excess" solvent microphase are thus $\Psi_S' = 0.095$ and $\Phi_W' = 0.513$ and the relative sugar content of the excess solvent is thus $\Psi'_s = 0.155$.

There is therefore a depletion of glucose from the small crystallites of lamellar phase present as multilayer vesicles in the dispersion, since the relative volume fraction of sugar inside the ordered phase is 0.095 while it is 0.155 in the excess "solvent". The initial global volume fraction of sugar $\Phi_S = 0.092$ corresponds to a relative fraction of 0.115 in the water.

The physical meaning of this depletion can be discussed in terms of non accessible water molecules strongly bound to the headgroups of the lipid. Since the water and lipid volume fractions in the lamellar phase are known, respectively 0.396 and 0.509, we can calculate the number n of water molecules per headgroup at maximum swelling in the presence of confined sugar:

$$n = (\psi_W/V_W)/(\psi_L/V_L) = 28$$

where V_W and V_L are the molecular volumes of water (30 Å^3) and of the fully hydrated lipid (1095 Å^3) known from the literature (Knoll, 1981). This value is to be compared to 25, the number of water molecules per headgroup in the absence of sugar.

In terms of molecular ratio, the swelling from 60.4 Å in absence of sugar to 69.8 Å is due to an increase of 3 water molecules and 1 sugar unit per headgroup.

4. Conclusion

The method presented here is quite general to determine partition coefficients of solutes in microphase separated samples, when two phases in coexistence cannot be separated. The scattering length density of the constituents, here common DMPC and partially deuterated glucose, do not need to be within the interval which can be matched by D_2O / H_2O mixtures. The only requirement is to be able to measure the contrast match point even if it has to be extrapolated outside the scattering length density range covered by H_2O/D_2O mixtures. Note that the global concentration of the host molecule – here the sugar – does not need to be known, it is not used at any step of the calculation. Since the scattering diverges at low q, it is important to check that in this *q*-range the CMP is q-independent thus avoiding data extrapolation to $q = 0$.

In the system presented here, detailed knowledge of the composition of the two coexisting phases will allow quantitative reinterpretation of the equation of state (pressure *vs.* distance) of zwitterionic model membranes in the presence of a hydrophilic component, susceptible of depletion as shown here, or in the opposite case of preferential adsorption. The equilibrium maximum swelling where attractive and repulsive interactions counterbalance, can now be interpreted, if the osmotic pressures are known. Complete description of the force balance explaining quantitatively the observed swelling and softening of the lamellar phase is in progress.

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