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Sugar does not affect the bending and tilt moduli of simple lipid bilayers

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ABSTRACT

The diffuse X-ray scattering method has been applied to samples composed of SOPC, DOPC, DMPC, and POPC with added sugar, either sucrose, glucose, fructose, maltose, or trehalose. Several sugar concentrations in the range 200–500 mM were investigated for each of the lipid/sugar samples. We observed no systematic change in the bending modulus K_C or in the tilt modulus K_θ with increasing sugar concentration. The average values of both these moduli were the same as those of the respective pure lipid controls within statistical uncertainty of 2%. These results are inconsistent with previous reports of sugar concentration dependent values of K_C.

1. Introduction

The bending modulus K_C is a fundamental mechanical property of membranes that has overarching biophysical relevance. It has been a concern (Nagle et al., 2015; Nagle, 2013) that, even for simple lipid bilayers, there are significant differences in the reported values for K_C. An hypothesis for the disparate values is that the true bending modulus may not only be a property of the intrinsic lipid bilayer but may also vary upon adding sugar to the aqueous environment. If true, this would affect the values of K_C determined by the classical methods of shape analysis (Meleard et al., 1998, 1997; Henriksen and Ipsen, 2002; Pecreaux et al., 2004; Gracia et al., 2010; Bouvrais, 2012; Vitkova and Petrov, 2013) and mechanical manipulation (Rawicz et al., 2000; Henriksen and Ipsen, 2004; Vitkova et al., 2006; Shchelokovskyy et al., 2011; Evans and Rawicz, 1990). Both methods have typically used sugar to improve optical contrast. The disparity in K_C values could then have arisen because different studies have used different sugar concentrations. In this report we test this hypothesis using the method of low angle diffuse X-ray scattering from oriented stacks of membranes to measure K_C (Lyatskaya et al., 2001; Liu and Nagle, 2004; Salditt et al., 2003; Li et al., 2006; Pan et al., 2008, 2009; Jablin et al., 2014). The most recent extension of this method also determines the tilt modulus K_θ (Jablin et al., 2014; Jablin, 2015), so we report the effect of sugar on this modulus that only our method has been able to determine experimentally. We conclude by briefly discussing why the shorter length scale probed by X-rays compared to the classical methods is unlikely to alter our conclusion that sugar has no effect on true bending moduli.

2. Experimental methods

Lipids studied were SOPC, DOPC, DMPC, and POPC obtained from Avanti Polar Lipids. Sugars studied were sucrose, glucose, fructose, maltose, and trehalose obtained from Sigma–Aldrich. Samples were made by first mixing lipid and sugar (first solubilized in heated trifluoroethanol or methanol) in excess 1:1 vol:vol chloroform/(trifluoroethanol or methanol) organic solvent. Mole ratios of sugar to lipid n_S are given in Table 1. The mixtures were then deposited on Si wafers using the rock and roll technique, creating stacks of about 2000 aligned bilayers (Tristram-Nagle, 2007). Dry samples were then hydrated in a humidity chamber *in situ* on the X-ray beamline. Hydration was conveniently even more rapid and, importantly, proceeded further with sugar than for pure lipid. Table 1 shows the repeat spacing D which contains a bilayer and its associated water. Previous studies obtained the mole ratio n_W of water/lipid for fully hydrated D spacings (Nagle and Tristram-Nagle, 2000). For samples with different D spacings, n_W was calculated, using the previously established result that the area per lipid remains the same within the investigated D spacing range (Nagle and Tristram-Nagle, 2000). Dividing the sugar/lipid ratio n_S by n_W gave the sugar/water ratio that is converted to aqueous sugar concentration C_S, listed in Table 1. This concentration is an average concentration of sugar in

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Table 1
List of lipid/sugar samples and their exposures at different repeat spacings D, corresponding to sugar concentrations C_S. Fully hydrated D values are indicated by *, and w estimates the relative goodness of the tilt-independent fit to the different exposures.

Lipid	Sugar	n_S	D (Å)	C_S (mM)	w
SOPC	None	0	65.8*	0	0.33
	Sucrose	0.21	65.0	428	0.26
			70.9	343	0.34
			70.5	348	0.31
	Glucose	0.22	68.6	395	0.49
			74.2	328	0.28
	Fructose	0.22	65.8	440	0.27
			73.8	332	0.15
DOPC	None	0	63.5*	0	0.33
	Sucrose	0.21	65.6	368	0.26
			69.3	323	0.29
	Fructose	0.21	65.9	364	0.13
			67.0	349	0.13
			62.0	425	0.29
	Maltose	0.17	64.4	316	0.75
			67.5	281	0.54
			70.0	259	0.69
			72.4*	241	0.70
	Trehalose	0.17	64.3	316	0.51
			67.6	259	0.65
			66.5	282	0.62
DMPC	None	0	62.7*	0	0.11
	Sucrose	0.21	65.5	357	0.32
	Glucose	0.19	63.3	412	0.08
			66.8	362	0.16
	Fructose	0.19	66.3	368	0.18
			67.3	355	0.31
POPC	None	0	65.1*	0	0.42
	Maltose	0.17	64.4	326	0.25
			68.6	280	0.37
			67.5	291	0.31
			69.6	271	0.37
	Trehalose	0.17	63.4	339	0.40
			65.9	308	0.27
			66.7	299	0.30

the water that includes both the water in the interfacial headgroup region and in the ample water space between neighboring bilayers in the well hydrated bilayer stacks.

X-ray scattering data were taken at G1 station at the Cornell High Energy Synchrotron Source (CHESS), following published protocols (Liu and Nagle, 2004). A sample was placed in a hydration chamber maintained at 30 °C, and it was hydrated through the vapor phase. All bilayers were in the fluid phase (relative humidity >99%) for all reported results. The X-ray wavelength was either 1.177 Å or 1.108 Å. During an X-ray exposure, the incident angle was continuously varied by rotating the sample between −1.6° and 7°. The lamellar repeat D spacing was increased by increasing the current through a Peltier cooler in contact with the bottom of the silicon wafer holding the sample; this cooling of the sample compared to the vapor increased the effective relative humidity at the sample. Diffuse scattering data were fit using a new analysis method that obtains both the bending modulus K_C and the tilt modulus K_θ as the parameters that provide the best fit to the measured intensity (Jablin et al., 2014; Jablin, 2015). The data were also fit with $K_\theta$fixed to a very large value, thereby effectively removing tilt from the analysis; these tilt-independent results for K_C agreed well with the earlier analysis method that did not incorporate tilt in the elasticity model (Liu and Nagle, 2004). Some samples were better fit than others; the inverse of the root mean residual sum of squares was used to assign a relative weight with values w shown in Table 1.

3. Results

Table 1 lists samples analyzed for this study. Most combinations of lipid and sugar were measured with several different values of the repeat spacing D in order to obtain several sugar concentrations C_S for the same sample. Usually, the sample was allowed to gradually become more hydrated, although some decreases in D were deliberately induced by manipulating the Peltier current. The time sequence for the exposures of each lipid/sugar sample followed the order shown in Table 1. For the controls with no sugar, the values for the D spacing shown in Table 1 are the fully hydrated values, D^*, that have been well established in these and previous studies (Nagle and Tristram-Nagle, 2000). For some of the sugar samples, it was verified that the largest reported D spacing was near its fully hydrated value D^*, although time did not permit accurate determinations of D^* for all samples. Nevertheless, we estimate for the concentrations of sugar shown in Table 1 that the fully hydrated D^* for the DMPC samples was about 69 Å, for DOPC samples D^* was about 73 Å, for POPC samples D^* was about 72 Å,

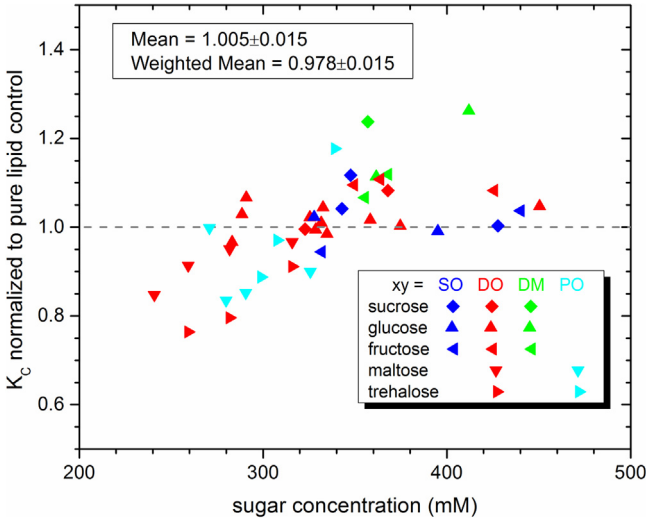

Fig. 1. Normalized K_C values for the combination of the sugars listed in the rows of the legend and for the xyPC lipids indicated in the columns of the legend. Normalization was to each pure lipid control. Values were obtained from tilt-independent fits. The DOPC/glucose results were previously published (Nagle et al., 2015).

and for SOPC samples D^* was about 75 Å. The important result is that for all cases the samples with sugar swelled to larger D values than the control with no sugar.

Fig. 1 shows results for the bending modulus K_C that were determined by fitting the data using the tilt-independent theory. To efficiently show the overall effect of all the sugars on all the lipids, the K_C for each lipid with sugar was divided by K_C for the pure lipid control. As emphasized earlier (Nagle et al., 2015), the DOPC/glucose ratios were all close to 1, with no systematic variations with sugar concentration C_S. Other results are more scattered. The smallest ratios are for DOPC/trehalose, but, contrarily, these trend to larger, not smaller, ratios with increasing C_S. The concentration dependence of trehalose appears different from the other sugars, as highlighted in Fig. S1 in supplementary content where it is noted that this result does not seem to relate to trehalose having special cryoprotectant properties. There is also no significant trend to smaller ratios with increasing C_S for the other lipid/sugar samples. The aggregate of DMPC ratios are greater than 1; this came about because the value of K_C for the pure DMPC control was smaller compared to previous results from this lab (Kucerka et al., 2005). Generally, our results for K_C for a single sample are subject to a standard deviation upwards to 10%, so up to 20% deviation in the ratios of two samples was expected. Within this expected uncertainty, there is no significant indication in Fig. 1 of any sugar having a different effect than the others. Averaging over all ratios for all combinations was done in two ways. The average that used the relative weights in the last column of Table 1 gives a mean ratio of 0.978 ± 0.015 where 0.015 is the estimated error of the mean, not the standard deviation. Averaging over all results with equal weights gives a mean ratio of 1.005 ± 0.015. Both averages are consistent with insignificant effect of sugar on K_C.

Fig. 2 shows similar results to Fig. 1 but for the tilt-dependent fits. The standard deviation of normalized K_C (0.153) is somewhat greater than for the tilt-independent fits (0.105), but the unweighted mean and the estimated error of the mean are consistent with the results of the tilt-independent fits.

The tilt-dependent analysis also determines the tilt modulus K_θ. Fig. 3 shows the ratios of K_θ with and without sugar. The relative standard deviation for K_θ is greater than for K_C as is expected because the diffuse scattering intensity is much more sensitive to the value of K_C than to K_θ. (However, tilt and bending play partially compensatory roles, so the larger uncertainty in K_θ lends an additional uncertainty to the values of K_C in the tilt-dependent fits; this partially accounts for the somewhat larger standard deviation in Fig. 2 compared to Fig. 1.) As with K_C, it appears that there is no systematic effect of sugar concentration on K_θ.

4. Discussion and conclusions

Our results show that there is no effect of common sugars on the bending modulus K_C or the tilt modulus K_θ of common lipids when determined using our X-ray scattering method. This is the first study of the effect of sugar on the tilt modulus K_θ.

Since our bending modulus result disagrees with some reported results using other methods (Nagle, 2013), let us discuss possible causes. The most obvious *a priori* cause for our not observing an effect might have been that sugar was not well incorporated into our samples, but was sequestered in regions where it did not significantly interact with the stacks of bilayers. Although we had limited control over this, fortunately, it was clearly not the case because sugar caused the repeat D spacing to increase dramatically. If the sugar had remained outside the stack of bilayers, then it would either have not affected the bilayers in which case D would have remained the same, or sugar would have competed with the bilayers for water, causing the D spacing to decrease. Another possible reason might have been that sugar could have been very strongly bound to the interfacial region so the average sugar concentrations we obtained would effectively have corresponded to negligible bulk concentrations in giant unilamellar vesicles used for the classical experimental methods. As discussed previously (Nagle et al., 2015), based on the partition coefficient of sugar between bulk water and bilayers (Andersen et al., 2011), the bulk water concentration is unlikely to be smaller by more than a factor of two. The average concentrations shown in Table 1 were chosen to be larger than most concentrations (10–300 mM) employed in giant unilamellar studies in order to ensure that, even if our effective concentrations were smaller than the nominal values by a factor of two, they would still be as large as those typically used.

The effect of sugar on the bending modulus as observed by the classical methods might be hypothesized as being due to K_C actually having different values on different length scales. Our X-ray analysis is more sensitive to shorter length scales of order 10–100 Å. In contrast, both classical methods assess longer length scales than the X-ray method; the length scale of the shape analysis method is of order of 10^4–10^5 Å, and the mechanical manipulation method averages from 10^5 Å down to 10 Å. We will now argue that

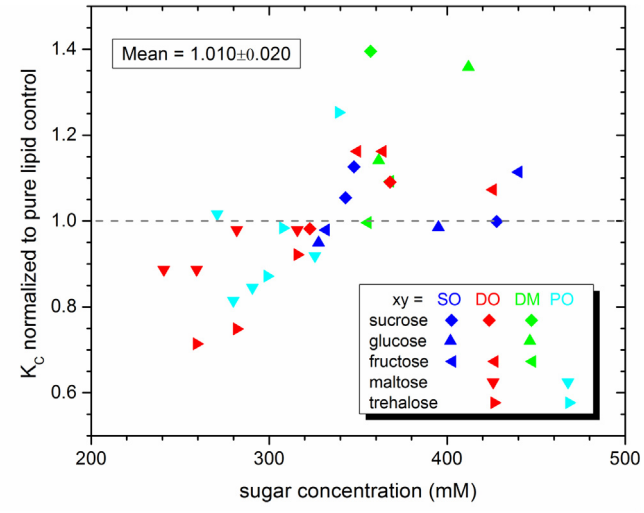

Fig. 2. Values obtained from the tilt-dependent fits for the normalized K_C values for the combination of the sugars listed in the rows of the legend and for the xyPC lipids indicated in the columns of the legend. Normalization was to each pure lipid control.

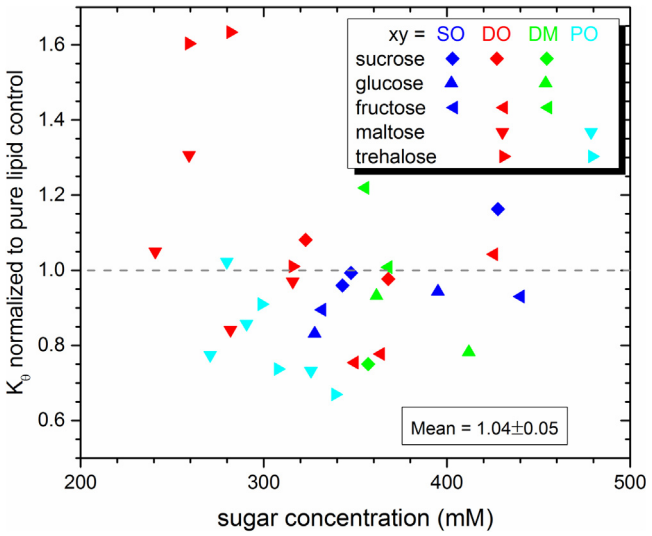

Fig. 3. Normalized values of the tilt modulus K_θ for the combination of the sugars listed in the rows of the legend and for the xyPC lipids in the columns of the legend. Normalization was to each pure lipid control.

our result that sugar does not affect the X-ray value of K_C is inconsistent with the hypothesis that K_C depends on the length scale. First, consider the ratios n_S of sugar molecules to lipid molecules given in Table 1; these correspond to about one sugar for five lipid molecules. This number of lipids has an interfacial area of approximately $5(65\,\text{Å}^2) = 325\,\text{Å}^2$, corresponding to a square of side length ~18 Å. This surface coverage of sugar is well characterized as homogeneous for the two classical methods, whereas it is within the range of X-ray sensitive length scales. Sugar could therefore have a different effect on the X-ray determined K_C values which we now discuss. Each sugar molecule might induce a local perturbation in the bilayer height profile that would surely be limited to 1 nm. The SA method looks at long wavelength thermal undulations and those have much larger amplitudes than 1 nm. Therefore, the local 1 nm perturbations just roughen those undulations on a length scale much shorter than the resolution limit of a typical optical microscope. In contrast, the shorter wavelength undulations assessed by X-rays have much smaller amplitudes. The putative local perturbations would substantially increase the relative amplitudes of these short wavelength modes and that increase would be captured by the X-ray analysis as a reduction in K_C even greater than a possible reduction due to sugar softening the bilayer. Our X-ray result of no reduction of K_C due to sugar is inconsistent with either type of reduction occurring. It therefore seems that the length scale hypothesis does not reconcile our X-ray results with previous reports that sugar reduces K_C.

The time scale is another difference between methods. Of course, K_C is an equilibrium property that therefore can not depend upon the time scale for systems which are in equilibrium, unless there is an artifact in the measurement method. The measured X-ray intensities are an ensemble average over many states. Each state is an average over the photon time scale of order femtoseconds, far too short for any significant temporal averaging of the sample to occur. Temporal averaging should also not be a concern for micromanipulation measurements which only assess the average area. However, it has been recognized to be a concern for the shape analysis method with its millisecond averaging (Faucon et al., 1989; Drabik et al., 2016). Temporal averaging smooths a vesicle's contour and such smoothing could be interpreted artifactually as larger values of K_C (The obvious extreme example is that if one waits long enough, the vesicle shape averages to a sphere which would correspond to an infinite value of K_C). Sugar increases the viscosity, thereby slowing down the fluctuations. For a given experimental apparatus with a fixed time scale, there would then be less temporal averaging with sugar, and if appropriate corrections were not made, the apparent value of K_C would appear smaller than its value without sugar, even if there were no real difference.

In conclusion, we find no effect of sugars on the bending modulus K_C or on the tilt modulus K_θ of neutral PC lipid bilayers using our X-ray method. In view of this, we urge further study using other methods to address whether there is an effect of sugar on the bending modulus.

Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemphyslip.2016.01.003.

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