BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Biochemistry **2009,** *48,* 11149–11151 11149
DOI: 10.1021/bi901662b

Buffers May Adversely Affect Model Lipid Membranes: A Cautionary Tale†

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Received September 23, 2009; Revised Manuscript Received October 22, 2009

ABSTRACT: The effects of biological buffers on lipids have not been fully investigated because of the long-standing assumption that these buffers are too hydrophilic to substantially interact with the lipid membrane. We present evidence that for some buffers, this is not necessarily the case. Our research points toward a membrane softening effect caused by the buffer molecules interacting with the headgroup region of the lipid. Changes in the elastic properties of the membrane are known to control membrane protein behavior; this work serves as a warning for the design of assays utilizing model membranes in the presence of buffers.

Lipids are able to spontaneously self-assemble into a wide variety of lyotropic mesophases. Lipids containing the phosphatidylcholine headgroup, such as 1,2-dioleoyl-*sn*-glycerophosphocholine (DOPC), are abundant in the membrane. In an aqueous solution, they form flat bilayers (the fluid lamellar, L_α, phase), which makes up the basic fabric of the biological membrane. In vivo, the cell membrane contains up to 1000 different lipid species, many of which do not by themselves form bilayers. The most ubiquitous of these are the phosphatidylethanolamines such as 1,2-dioleoyl-*sn*-glycerophosphoethanolamine (DOPE) in which the lipids form cylindrical monolayers that pack onto a two-dimensional hexagonal lattice, the inverse hexagonal, H_{II}, phase (*1*). The driving force behind the formation of such nonbilayer structures is the propensity of the lipid within a monolayer to curve toward the water. By adjusting the relative concentrations of bilayer and nonbilayer lipids in a membrane, cells regulate the membrane-stored curvature elastic stress homeostatically (*2*−*5*). Model systems based on phospholipid vesicles have been widely used as simple approximations of the cell membrane in a variety of biochemical and biophysical assays. While the naturally occurring lipid membrane of cells is a heterogeneous, dynamic environment comprised of thousands of different lipid species, lipid vesicles are, in general, made from simple lipid mixtures and can be used to elucidate the effects of various mechanical properties of lipids on the behavior of membrane proteins. In particular, they can be used to examine the effect of stored curvature elastic stress within the lipid membrane. Mounting evidence suggests that a range of cellular processes are sensitive to stored curvature elastic stress, including peripheral membrane proteins that catalyze the modification, synthesis, and degradation of membranes (*3*), mechano-sensitive channels (*6*), and the folding of integral membrane proteins (*7*).

Both model lipid systems and biological membranes are subject to a variety of attractive and repulsive forces that govern their shape and interactions with other membranes and whose mathematical descriptions are well understood (*8*). When lipid bilayers self-assemble in water, they do so with a repeat spacing characteristic of the lipid bilayer thickness and a water layer thickness between the bilayers. The sum of these measurements is the characteristic *d* spacing that can be measured by small-angle X-ray scattering (SAXS). Between bilayers there is a long-range attractive van der Waals force, the presence of which accounts for the fact that in the presence of water, bilayers do not swell indefinitely and when hydrated beyond their excess point exist in equilibrium with water. Bilayers in a fluid state, such as those that make up the fluid lamellar (L_α) state of the biological membrane, will undergo thermal undulatory motion leading to repulsive forces as the undulating membranes approach each other. It is known that the insertion of small molecules into the headgroup region of membranes causes softening of the membrane (*9*−*11*), leading to an increased level of undulation, and affects the stored curvature elastic stress contributions to the overall free energy of the membrane. In particular, the undulatory repulsion is inversely proportional to the bending modulus, whereas the stored curvature elastic energy is directly proportional to it (*12*, *13*).

Recently, studies on the refolding of diacylglycerol kinase (DGK) from *Escherichia coli* found that the mixture of 98:2 DOPC/DOPE vesicles suspended in a buffer mixture required for protein activity [DGK buffer, consisting of 75 mM PIPES, 0.1 mM EDTA, 50 mM LiCl, and 15 mM $MgCl_2$ (pH 6.8)] gave anomalously high refolding yields and rates of refolding (*14*). These rates could not be explained by a linear increase in curvature elastic stress which was predicted for the addition of this small amount of nonbilayer lipid. To investigate this further, we sought to decouple the effects of salts and buffer on the lipid membrane and systematically vary each component of the assay mixture to elucidate its role in membrane modification. We present here the effects of buffer alone on the swelling behavior of DOPC, DOPE, and 98:2 DOPC/DOPE vesicles as measured by SAXS. Commonly used biological buffers such as phosphate-buffered saline (PBS), Tris, HEPES, and PIPES were investigated at varying concentrations to ascertain whether the buffer alone played any role in altering the structural properties and dimensions of the lipid bilayer. We prepared samples by codissolving lipids at the desired ratio in cyclohexane followed by flash-freezing in liquid N_2, after which they were lyophilized for up to 24 h. Approximately 3 mg of lipid was added to an X-ray capillary (Gulmay Medical Ltd., 1.5 mm diameter) and rehydrated with 60% (w/w) buffer or salt solution to ensure the aqueous phase was in excess. Further details can be found in the Supporting Information. The capillary was then centrifuged at

†T.P.-S. was supported by an Erasmus exchange programme from the University of Valencia, Bancaja Erasmus, and Valencia City Hall. A.M.S. was supported by an EPSRC LSI Fellowship and EPSRC Platform Grant EP/G00465X/1.

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Published on Web 10/27/2009

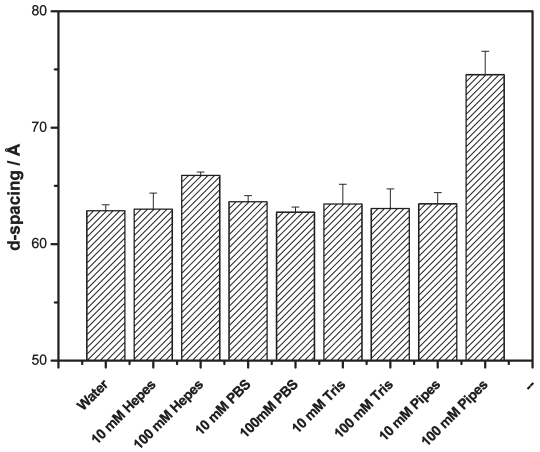

FIGURE 1: Calculated *d* spacing for 98:2 DOPC/DOPE vesicles in each buffer.

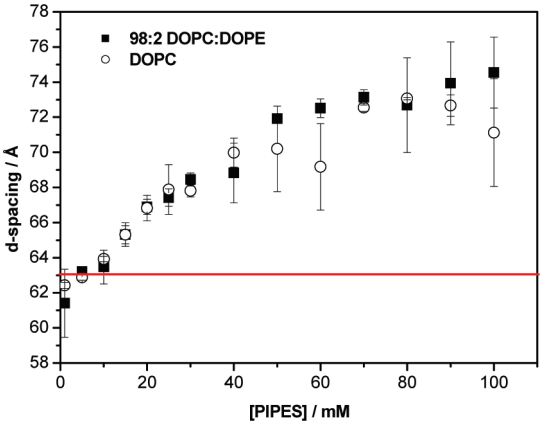

FIGURE 2: Change in *d* spacing with an increasing PIPES concentration for 98:2 DOPC/DOPE vesicles and DOPC alone. The red line indicates the expected *d* spacing of the sample hydrated in pure water.

2800 rpm for 40 s to mix, followed by five freeze−thaw cycles, after which the capillary was flame-sealed and equilibrated at room temperature for 24 h.

SAXS measurements were taken using a specialized in-house SAXS/WAXS beamline at 25 °C. Full details of the experimental design are given in the Supporting Information. On average, five exposures of 60 s were taken and added per image. A minimum of three separate samples were measured per data point shown. Data were analyzed using an in-house IDL-based software package, AXcess (*15*). X-ray measurements were calibrated with silver behenate (d_{001} = 58.38 Å).

When they were hydrated in water, the SAXS pattern of 98:2 DOPC/DOPE vesicles shows the expected L_α phase behavior and a *d* spacing of 62.87 ± 0.50 Å. The L_α phase was also observed for all samples measured in buffer. Figure 1 shows the *d* spacings obtained in each buffer (and water). The *d* spacings in 10 mM PBS, 100 mM PBS, 10 mM Tris, 100 mM Tris, and low concentrations (10 mM) of HEPES and PIPES correspond within error to the value for the L_α phase *d* spacing in water. However, samples hydrated in 100 mM HEPES showed an increase in the *d* spacing to 65.91 ± 0.27 Å, while those hydrated in 100 mM PIPES were found to have a *d* spacing increased to 74.54 ± 2.02 Å. Data for pure DOPE showed the presence of an inverse hexagonal (H_{II}) phase that did not undergo PIPES-dependent swelling; the data are given in the Supporting Information.

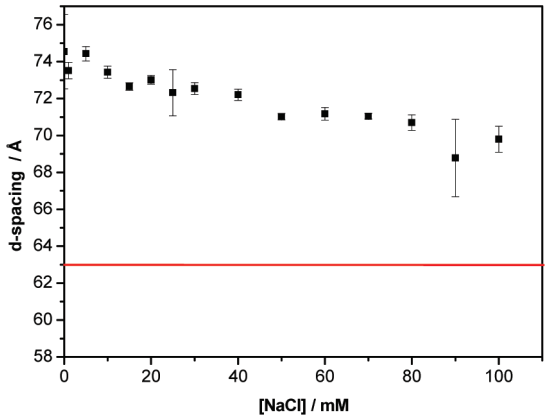

FIGURE 3: Change in the *d* spacing of 98:2 DOPC/DOPE vesicles with the addition of an increasing concentration of NaCl. The red line indicates the expected *d* spacing in pure water.

Figure 2 shows SAXS measurements for the *d* spacing of 98:2 DOPC/DOPE vesicles and DOPC alone with increasing concentrations of PIPES, demonstrating that the increase in *d* spacing was dependent on PIPES concentration. To investigate whether this swelling was merely due to electrostatic repulsion of bilayers containing charged PIPES molecule interacting with the headgroup region of the membrane without any other mechanical effects on the membrane, SAXS measurements of 98:2 DOPC/DOPE vesicles in 100 mM PIPES with increasing concentrations of NaCl were taken and are shown in Figure 3. The *d* spacing can be seen to decrease upon addition of salt but never returns to the values obtained for a sample in pure water. The SAXS results presented here show that commonly encountered biological buffers such as PIPES and HEPES can affect the swelling behavior of a lipid bilayer, which may have implications for their usage in in vitro biochemical assays, in particular those that rely on the physical properties of the lipid membrane as a parameter. PIPES and HEPES are two members of the class of biological buffers known as The Good's buffers (*16*), developed in the 1960s to adhere to criteria deemed to be important for biochemical and cell biology assays. These criteria include a pK_a between 6 and 8, maximum solubility in water compared to other substances, and a weak ability to pass through biological membranes. PIPES is zwitterionic, with a pK_a value of 6.66 at 37 °C. It has found use in applications as a preserving buffer when used with aldehyde fixatives for electron microscopy (*17*−*20*). However, membrane alterations such as artifactual multivesicular myelin fingers in samples of rat cerebral cortex as measured by transmission election microscopy have been observed in the presence of PIPES buffer (*20*) which were not seen when cacodylate or phosphate buffer was used, pointing to a potential interaction between PIPES molecules and the lipid membrane. From the results presented here, it appears that PIPES (and, to a lesser degree, HEPES) has an effect on bilayer spacing in model lipid systems. The increase in *d* spacing and the classic swelling curve behavior seen when DOPC (and 98:2 DOPC/DOPE) is hydrated with PIPES suggests that the buffer molecules are causing an increase in the *d* spacing by either intercalation in the headgroup region of the lipid bilayer, leading to membrane softening, or electrostatic repulsive effects. We have added NaCl to this system to separate the effect of charge and softening on the buffer-induced increase in *d* spacing (Figure 3).

The data demonstrate that only a small part of the observed increase in *d* spacing is due to any electrostatic repulsion caused by the buffer at the membrane interface. At 100 mM NaCl, the system is at a 1:1 molar ratio of buffer to NaCl and the salt screening means the Debye length is 9 Å. One would therefore anticipate that the majority of any electrostatic repulsion would have vanished, yet the *d* spacing appears to have stabilized to a spacing that is approximately 7 Å greater than that without buffer. Thus, the role of the buffer molecules in the swelling behavior seems to go beyond that of simple electrostatics. An alternative (and mutually inclusive) hypothesis is that buffer intercalation within the headgroup region of the bilayer has reduced the membrane bending modulus and led to an increase in number of fluctuations of the membrane. It is possible that PIPES has a greater effect on the membrane compared to HEPES as PIPES is less water-soluble; its increased hydrophobicity would lead to it being more likely to go to the lipid interface and cause swelling.

This evidence of an increased number of membrane fluctuations tells us that the stored curvature elastic energy in the membrane has been reduced. In studies where the activity or dynamics of a protein are modulated by stored curvature elastic stress, the use of PIPES or high concentrations of HEPES will mask or distort such effects.

Given our understanding of the role of biomechanical control of the lipid membrane in the function of membrane proteins (*21*), these preliminary data should strike a note of caution to those wishing to undertake experiments in model systems in which the structural properties of the lipid bilayer are a factor. Work is continuing in our laboratory to characterize the generality of this phenomenon and its potential impact on the success of biochemical assays.

SUPPORTING INFORMATION AVAILABLE

A summary of the materials used, methods of sample preparation, and X-ray equipment used. This material is available free of charge via the Internet at http://pubs.acs.org.

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