Journal of Biomolecular NMR, **12:** 447–451, 1998.
KLUWER/ESCOM

Improved dilute bicelle solutions for high-resolution NMR of biological macromolecules

Judit A. Losonczi and James H. Prestegard*
Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Rd., Athens, GA 30602-4712, U.S.A.

Received 22 April 1998; Accepted 1 June 1998

Key words: bicelle, charged lipids, DLVO theory, liquid crystal NMR, magnetic orientation

Abstract

Dissolving biological macromolecules in dilute bicelle solutions, which form oriented liquid crystals in the presence of a magnetic field, permits measurement of anisotropic spin interactions such as dipolar couplings [Tjandra, N. and Bax, A., *Science*, **278**, 1111–1114]. However, the lifetimes and temperature ranges of orientation for these samples are critically dependent on sample composition and experimental conditions. This paper demonstrates that doping dilute bicelle solutions with small amounts of charged amphiphiles substantially improves the stability and degree of alignment, as well as extends the temperature range of orientation for these systems. An explanation of the dependence of bicelle aggregation on sample composition is proposed based on the DLVO theory of colloids.

Liquid crystalline media have long been used to orient small solutes for study by NMR methods (Emsley and Lindon, 1975). Even partial orientation allows direct measurement of anisotropic interactions that normally average to zero in solution NMR, such as residual dipolar couplings and chemical shift anisotropies (CSAs). In principle measurement of these interactions can be extremely valuable in structural studies of both small and large molecules. The dipolar couplings depend on both the distance between the two nuclei and the angle of the internuclear vector with respect to the magnetic field. The chemical shift offsets that result from CSAs depend on the orientation of a molecule fixed shift tensor with respect to the magnetic field. If measurements of these interactions can be made on larger molecules and converted into distance and orientational constraints it might be possible to improve the accuracy and range of molecular structures determined by NMR methods.

A few years ago applications of liquid crystal NMR techniques were extended to some larger, biologically relevant molecules with the aid of isotopic labeling and a medium comprised of an aqueous dispersion of lipid bilayer disks (most commonly a mixture of dimyristoylphosphatidylcholine and dihexanoylphosphatidylcholine, DMPC and DHPC) (Sanders et al., 1994). These disks are now referred to as 'bicelles' (Sanders and Landis, 1995). Applications had been primarily to molecules which orient very strongly because of direct association with the bicelle, for example membrane associated proteins and peptides. These molecules exhibit large residual interactions that have required use of at least some solid NMR methodology (Howard and Opella, 1996; Rinaldi et al., 1997; Losonczi and Prestegard, 1998). Recently, more general applications to soluble proteins have proven possible (Tjandra and Bax, 1997; Bax and Tjandra, 1997). By diluting the bicelle medium and carefully controlling the temperature of observation, Bax and colleagues have been able to elicit a low degree of order for water soluble proteins, a degree of order that results in small interactions that mesh well with traditional high resolution NMR methodology. It is this advance that offers the promise of widespread applications to solution studies of macromolecules.

*To whom correspondence should be addressed.

The use of dilute bicelle solutions to orient different macromolecules is, however, not problem free. Even at low concentrations of solutes the samples often phase separate or become isotropic in times short compared to normal NMR data acquisition times. Instabilities seem to be aggravated by the presence of buffers and electrolytes needed to maintain native structures of some biomolecules (Yang et al., 1998). This paper presents evidence that the addition of small amounts of charged amphiphiles to normal bicelle preparations can avoid some of these instabilities and extend the range over which these preparations maintain a useful degree of orientation and homogeneity.

The alignment of bicelle preparations can be studied by observing anisotropic interactions such as chemical shift anisotropy (CSA) offsets, dipolar couplings, or quadrupolar couplings that originate from the molecules that actually comprise the medium. For example, the ^{31}P NMR spectrum of a bicelle solution reflects the average orientation of the phosphate headgroup's CSA tensor relative to the magnetic field (Figure 1) (Kohler and Klein, 1977). When the system is isotropic, the anisotropic interactions average to zero, yielding a single ^{31}P resonance close to zero ppm. Since the bicelles orient with their bilayer normal perpendicular to the magnetic field (Sanders et al., 1994) and the headgroups are oriented more or less as depicted in Figure 1, orientation results in the phosphorus resonances shifting upfield. Water in the medium provides another probe of orientation if some water protons are replaced with deuteriums. Deuterium has a quadrupolar nucleus that interacts with both the laboratory magnetic field and a local electric field gradient. The electric field gradient interactions add an anisotropic perturbation that splits the normally degenerate resonances of a deuterium nucleus. Again, under isotropic tumbling these splittings average to zero. In the presence of oriented bicelles, a small quadrupolar coupling results from the fast exchange of bulk solvent, which is isotropic, and the solvent associated with the bilayer, which is partially oriented. Both ^{31}P and ^{2}H NMR thus provide monitors of the behavior of bicelle media in the presence of a magnetic field.

Experiments were carried out on a 500 MHz Varian Inova spectrometer equipped with an HX double resonance probehead. For ^{31}P experiments the X channel was used with a 20 kHz ^{1}H decoupling field. ^{2}H spectra were collected using the lock channel and for these experiments no proton decoupling was used. Spectra as a function of temperature were acquired by starting

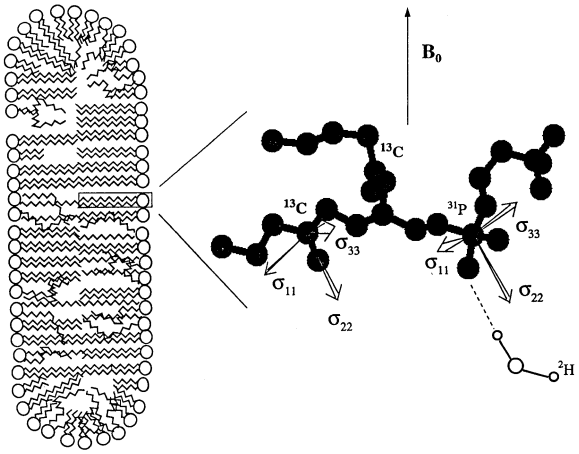

Figure 1. Orientation of the phosphate headgroup's CSA tensor relative to the magnetic field in an oriented bicelle solution.

at 25 °C and then raising the temperature by two degrees at each step. Samples were allowed to equilibrate at the desired temperature for at least 10 min prior to acquisition of NMR data.

The samples contained a mixture of DMPC (Sigma, St. Louis, MO) and DHPC (Avanti Polar Lipids, Birmingham, AL). Samples were prepared based on a method suggested for higher lipid concentration (25% w/v) bicelle samples (Howard and Opella, 1996). The DHPC was dissolved in a small volume of buffer (clear solution), while the DMPC was also mixed with a small amount of buffer giving a white dispersion. Vortexing and several freeze/thaw cycles made the DMPC dispersion homogeneous. This was then warmed above the T_m of pure DMPC (23 °C) and the DHPC solution was added to it. The solution was briefly vortexed and then quickly frozen in a methanol/solid CO_2 bath. The solution was then allowed to slowly come to room temperature. Since DHPC is very hygroscopic, the exact DMPC:DHPC molar ratio cannot be reliably estimated by weighing out dry DHPC and DMPC, so this molar ratio was determined by integration of the ^{31}P spectrum and found to be 3.2:1. Bicelle systems with slightly different DMPC:DHPC ratios were also tested. By lowering the DMPC:DHPC ratio the range of temperatures over which orientation is maintained is shifted slightly higher. All samples contained 5% w/v lipid in aqueous buffer with 15% $^{2}H_2O$ in place of H_2O. Buffers were made from commercially available chemicals. The charged amphiphiles which we added to the normal bicelle preparations, hexadecyl(cetyl)trimethylammonium bromide (CTAB) and sodium dodecyl sul-

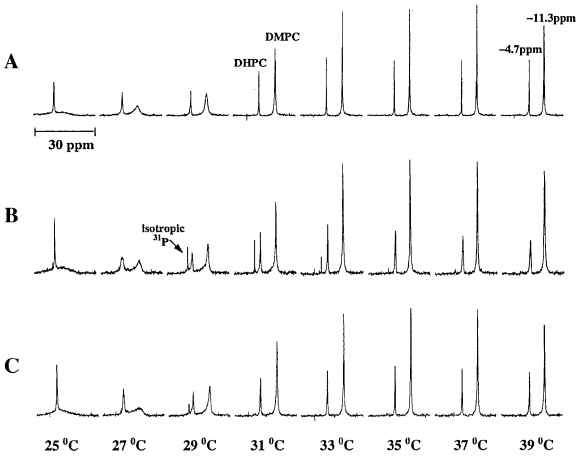

Figure 2. Changes in the ^{31}P NMR spectra of different bicelle solutions upon increasing temperature. A: 3.2:1 DMPC:DHPC, 5% w/v lipid in water. B: 3.2:1 DMPC:DHPC, 5% w/v lipid in 50 mM pH 6 BisTris buffer. C: 3.2:1:0.1 DMPC:DHPC:CTAB, 5% w/v lipid in 50 mM pH 6 BisTris buffer.

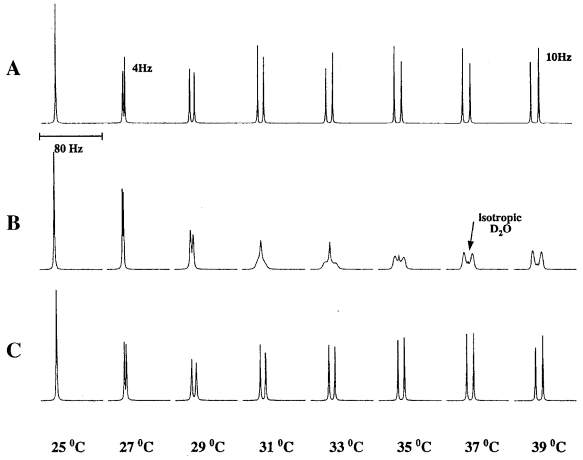

Figure 3. Changes in the ^{2}H NMR spectra of different bicelle solutions upon increasing temperature. A: 3.2:1 DMPC:DHPC, 5% w/v lipid in water. B: 3.2:1 DMPC:DHPC, 5% w/v lipid in 50 mM pH 6 BisTris buffer. C: 3.2:1:0.1 DMPC:DHPC:CTAB, 5% w/v lipid in 50 mM pH 6 BisTris buffer.

fate (SDS) were obtained from ACROS (Fair Lawn, NJ) and Sigma, respectively. They were added to bicelles in a small volume of concentrated solution, followed by vortexing and temperature alternation.

Figures 2 and 3 compare changes in the ^{31}P and the ^{2}H spectra upon increasing temperature for three different samples. Figures 2A and 3A show the behavior of a 5% bicelle solution made with pure, deionized water, which we consider to be a standard bicelle preparation. At 25 °C the sample is isotropic, and both ^{31}P and ^{2}H spectra show single lines, one for deuterated water in the ^{2}H spectrum, one for each of the phospholipids in the ^{31}P spectrum (the DMPC line is broad). Upon increasing the temperature the sample becomes aligned in the magnetic field. At 27 °C the sample is weakly oriented; the deuterium signal is a doublet but the splitting is small. The phosphorus resonance of the DMPC is still quite broad but is noticeably upfield shifted. At this temperature the sample is cloudy but seems to be homogeneous by NMR criteria. By 35 °C the sample becomes clear and well ordered; it remains so to temperatures of 40 °C and more.

The behavior of a 5% bicelle solution made with a 50 mM BisTris ([Bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane) buffer is very different, as illustrated in Figures 2B and 3B. At 25 °C the sample is isotropic, as in Figures 2A and 3A, and with increasing temperature it shows signs of weak orientation. However, even at 29 °C there are signs of heterogeneity. The ^{31}P spectrum shows a small isotropic peak in addition to the oriented spectrum. At 31 °C the ^{2}H spectrum also shows a central isotropic component superimposed on the oriented doublet. This can only arise if two phases exist with sufficient separation to make water diffusion between the phases slow on the NMR timescale. The bicelles probably form a higher lipid concentration oriented liquid crystal in the lower part of the NMR tube while the top part of the sample is mainly isotropic water. This phase separation is visible when the sample is taken out of the magnet and is only reversible with remixing. Similar experiments were performed using 50 mM pH 6 phosphate buffer and they gave comparable results. Using 25 mM buffer shifts the temperature of the phase separation a few degrees higher. The behavior may be somewhat pH dependent, but the primary cause is more likely connected to ionic strength. Analogous experiments were performed with a 5% bicelle solution made with 100 mM KCl solution. This sample also phase separated in the 30–40 °C temperature range.

The effects of ionic strength on what appears to be bicelle aggregation are very reminiscent of those on the aggregation of other bilayer and micelle systems (Degiorgio and Corti, 1985). There are two major forces that act between surfaces of lipids and influence interactions, such as aggregation of bilayers. Van der Waals forces occur between all solute molecules in water. At long distances these forces are attractive (Israelachvili, 1973). If the interacting surfaces

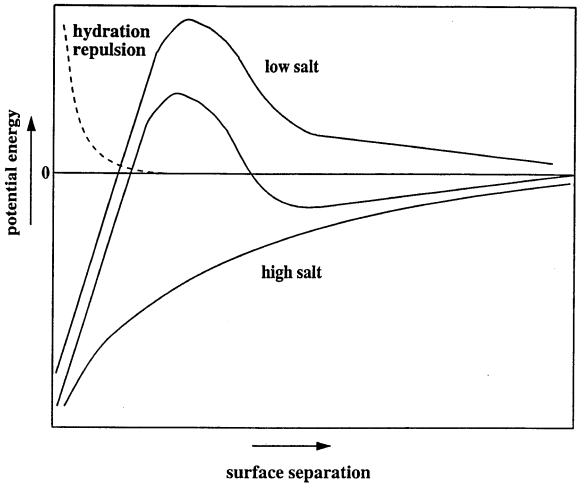

Figure 4. Potential energy between two charged surfaces as a function of surface separation that would be expected based on DLVO theory.

possess like partial charges they also experience a repulsive electrostatic force. The total potential energy of interaction can be determined by addition of the repulsive and attractive potential energies. Figure 4 shows qualitatively the potential energy of interaction between two charged surfaces as a function of surface separation based on DLVO theory (Verwey and Overbeek, 1984). There is always a hydration term as well as the repulsive part of the Van der Waals interaction that keeps bilayers from falling into the very short distance minimum and completely collapsing. However, a secondary minimum can arise at longer distances under certain combinations of electrostatic and Van der Waals terms. Since the electrostatic term is modulated by ionic strength, these minima occur only above certain ionic strength levels (or certain salt concentrations). It is these minima that are responsible for micelle and bilayer aggregation. Of course, the bilayer or micelle must be inherently charged before this ionic strength dependent explanation pertains. However, in the absence of charge there can be minima which result from the interplay of hydration and Van der Waals forces that also lead to aggregation. In fact, neutral lipid bilayers have an inherent tendency to aggregate into multilayer particles.

So, now the question arises: Can dilute, supposedly neutral bicelles be charged, and can this contribute to stability in deionized water? Commercially available DMPC and DHPC are only around 99% pure and impurities can be charged. Furthermore, any additional hydrolysis of phospholipids produces either a charged lipid or a partially ionized fatty acid. Thin layer chromatography and mass spectrometry showed minor contamination in our DMPC that was consistent with being lyso-DMPC. The released fatty acid could contribute an amount of surface charge sufficient to stabilize 'neutral' bicelles in water. If this explanation is correct it should also be possible to manipulate charge artificially to improve stability in the presence of moderate ionic strength buffers.

The effect of doping the bicelles with a charged lipid like CTAB at a DMPC:CTAB ratio of 30:1 in the presence of 50 mM BisTris is shown in Figures 2C and 3C. As for the previous samples, at 25 °C the medium behaves isotropically. As the temperature is increased the sample goes trough a phase transition, becoming weakly oriented and cloudy. But unlike the non-doped bicelles, at higher temperatures the sample doesn't phase separate; it clears and becomes highly and uniformly oriented. DMPC:CTAB ratios of 100:1, 50:1 and 30:1 were tested. A DMPC:charged lipid ratio of 50:1 seems adequate to avoid phase separation in the case of a 50 mM salt solution, but this number is highly dependent on the specific sample and the concentration of the buffer. The effect of a negatively charged lipid, SDS, was also tested and showed very similar effects to those of CTAB.

This paper demonstrates a possible advantage in doping dilute bicelle systems with small amounts of charged lipid. The gain is twofold. The presence of charged lipids compensates for the destructive effect of high salt concentrations on bicelle orientation and sample lifetime. Also, using charged lipids can in principle help minimize interactions between the macromolecules to be studied and the bicelles. One simply chooses a lipid charged in a like-manner to that of the macromolecule at the solution pH. This proposed method has been used successfully in the case of several positively charged proteins with CTAB containing bicelles (a protein fragment from barley lectin (Weaver and Prestegard, 1998), ubiquitin (Yang et al., 1998), and DnaJ (Huang et al., 1998)), as well as a highly negatively charged carbohydrate (GXM) with SDS containing bicelles. Thus, the bicelle compositions described are experimentally useful. More varied applications in the future should test the validity of our proposed explanation based on DLVO theory.

Acknowledgements

We thank Steven Levery at the Complex Carbohydrate Research Center in Athens, GA for help with the thin layer chromatography and the mass spectrometry. We would also like to thank R. Scott Prosser at the University of Kent, OH for kindly providing us with a figure of a bicelle. This work was supported by grants from the National Institutes of Health, GM54160 and GM33225.

References

Bax, A. and Tjandra, N. (1997) *J. Biomol. NMR*, **10**, 289–293.

Degiorgio, V. and Corti, M. (1985) *Physics of Amphiphiles: Micelles, Vesicles and Microemulsions*, North-Holland Physics Publishing, Amsterdam.

Emsley, J.W. and Lindon, J.C. (1975) *NMR Spectroscopy Using Liquid Crystal Solvents*, Pergamon Press, Oxford, UK.

Howard, K.P. and Opella, S.J. (1996) *J. Magn. Reson.*, **B112**, 91–94.

Huang, K., Flanagan, J. and Prestegard, J.H. (1998) *Protein Sci.*, in press (description of the protein only).

Israelachvili, J.N. (1973) *Q. Rev. Biophys.*, **6**, 341–387.

Kohler, S.J. and Klein, M.P. (1977) *Biochemistry*, **16**, 519–526.

Losonczi, J.A. and Prestegard, J.H. (1998) *Biochemistry*, **37**, 706–716.

Rinaldi, F., Lin, M.F., Shapiro, M.J. and Petersheim, M. (1997) *Biophys. J.*, **73**, 3337–3348.

Sanders, C.R., Hare, B.J., Howard, K.P. and Prestegard, J.H. (1994) *Prog. NMR Spectrosc.*, **26**, 421–444.

Sanders, C.R. and Landis, G.C. (1995) *Biochemistry*, **34**, 4030–4040.

Tjandra, N. and Bax, A. (1997) *Science*, **278**, 1111–1114.

Verwey, E.J.W. and Overbeek, J.T.G. (1984) *Theory of the Stability of Lyophobic Colloids*, Amsterdam.

Weaver, J.L. and Prestegard, J.H. (1998) *Biochemistry*, **37**, 116–128 (description of the protein only).

Yang, D., Tolman, J. R., Goto, N. K. and Kay, L. E. (1998) *J. Biomol. NMR*, **12**, 325–332.