Chapter 2

Media for orienting biological macromolecules

Franciska van Lune and Ruud Scheek
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Abstract

Residual dipolar couplings, which become measurable at small degrees of alignment of molecules in a magnetic field, turn out to be extremely useful parameters for protein structure determination and refinement. The stability of the orienting media used for aligning biomolecules varies with sample composition and experimental conditions. Until now, no universally applicable medium has been presented. In this review we give an overview of the properties of the orienting media used, including their advantages and disadvantages. In addition some suggestions and practical tips for the preparation of the different media will be given. Table 1 at the end of this review gives an overview of the properties of the media that are discussed.

Introduction

Anisotropic interactions such as the magnetic dipole-dipole interaction are often used in solid-state NMR experiments to obtain structural information. However, in liquid state NMR these interactions average out to zero due to the rapid and isotropic tumbling of the macromolecule in solution. By adding a liquid crystalline medium biological macromolecules are prevented from tumbling entirely isotropically and consequently small residual dipolar couplings can be measured (Tjandra & Bax, 1997).

The mechanism of alignment of the liquid crystalline media relies on the interaction between the magnetic field and the magnetic susceptibility of the molecules. When a molecule is placed in a magnetic field, a magnetic dipole moment is induced which is proportional to the susceptibility, $\chi$. Upon placement of diamagnetic molecules in a magnetic field, the induced moments will oppose the external magnetic field ($\chi<0$), whereas paramagnetic molecules ($\chi>0$) will induce moments which are along the field. The induced moments will interact with the magnetic field in a way that is orientationally dependent because of the anisotropic distribution of electrons in the molecules. The interaction energy, $W$, can be written as follows:

$$W = \frac{1}{\mu_0} \left( -\frac{1}{2} \chi B^2 \right)$$

where $B$ is the magnetic field strength, $\chi$ is the magnetic susceptibility tensor and $\mu_0$ is the vacuum permeability. Only if the variations in $W$ that arise from the anisotropy in $\chi$ are large enough compared with the thermal energy, $kT$, will a measurable degree of orientation be induced (Prestegard et al., 2000).

Some biological macromolecules have sufficiently large susceptibility anisotropies of their own and consequently can be directly aligned in a magnetic field. Usually, however, the macromolecule is dissolved in an aligned medium (Prestegard & Kishore, 2001). Tjandra and Bax (1997) used a mixture of dimyristoyl phosphatidylcholine (DMPC) and dihexanoyl phosphatidylcholine (DHPC) as alignment
medium (figure 1). After their breakthrough a range of other liquid crystalline media has been demonstrated to achieve the desired weak degree of alignment.

There are several reasons for employing more than one orienting medium. First, not all orienting media are compatible with the macromolecule’s solubility requirements (e.g. pH, temperature, ionic strength). Second, different alignment media might result in different orientations of the molecule under investigation with respect to the magnetic field (Gronenborn, 2002). If the orientation of the alignment tensors is different in the orienting media used, the residual dipolar coupling data obtained can be combined to restrain a given interatomic vector to the intersection of the sets of possible orientations (de Alba & Tjandra, 2002).

The final orientation of the molecule of interest depends on i) the alignment of the orienting medium with respect to the magnetic field (parallel or perpendicular) and ii) the mechanism of orientation (steric and/or electrostatic interactions). Steric interactions can be described as an obstruction effect between the macromolecule and the medium. In this case, it is the shape anisotropy of the molecule that dictates the orientation. In case of electrostatic interactions the orientation of the macromolecule depends on the electrostatic charge distribution in the medium and the molecule. This charge varies with the pH and ionic strength of the solution as well as with the pKa’s of the molecules involved (Burnell & de Lange, 2003).

Many orienting media have been published over the last few years, but none is universally applicable. An appropriate magnetically orienting medium must fulfil at least the following conditions (Brunner, 2001; Clore & Gronenborn, 1997):

1. The orienting medium must remain stable and oriented when added to a protein solution. It should not lead to phase separation.

2. Addition of the medium must not result in chemical or structural changes of the macromolecule under study. Therefore the macromolecule should not interact strongly with components of the medium.
3. The medium must be able to achieve a weak level of alignment (typically < 10^{-3}) for the molecule of interest. Too much alignment results in an exponential increase in the number of observed dipolar splittings with a concomitant large decrease in sensitivity and resolution.

4. It must be miscible with water.

Whether a particular orienting medium meets these requirements cannot always be predicted a priori and it is often necessary to empirically determine which medium is best suited for a specific case. In general, proteins that interact with membranes as well as flexible and partially folded proteins are not compatible with phospholipid-based media. In addition, negatively charged molecules interact with positively charged media and vice versa (Gronenborn, 2002).

In this review the properties, advantages and drawbacks of the different alignment media will be discussed to make it easier to find the most compatible media. Also some suggestions for the preparation of the media will be given.

**Liquid crystalline media based on phospholipids**

The first liquid crystalline medium used for the measurement of residual dipolar couplings is the phospholipid based medium consisting of mixtures DMPC and DHPC (Tjandra & Bax, 1997). Initially these mixtures where believed to form disc-shaped phospholipid bilayers, so called bicelles with diameters of several hundred Å and thicknesses of ~40 Å. According to this model the long chain phospholipid, DMPC, mainly forms the bilayer part of the bicelle with the detergent, DHPC, surrounding the edges (figure 2). It was believed that these bicelles were formed just above the temperature (~27 °C) where the lipid acyl-chains switch from a gel to a liquid crystalline phase.

![Figure 2: Sketch showing the structure of a phospholipid bicelle, formed by a mixture of the phospholipids DMPC and DHPC](image)

In 2001, Gaemers and Bax (Gaemers & Bax, 2001) proposed a new model. Their data show that the phospholipids do form bicelles. However, these particles are formed
below the melting temperature of DMPC instead of above the transition temperature. At higher temperatures, where the medium is liquid crystalline, they suggest edge to edge interactions between the disks. This makes it equivalent to strongly perforated lamellae with DHPC lining the rims of the holes, so called bilayered Swiss cheese sheets (figure 3a). Others suggest a structure like Swiss-cheese sheets as well (Nieh et al, 2001; Nieh et al, 2002; Prosser et al, 1998a; Wang et al, 2003). Electron microscopy experiments performed in house confirm the model proposed by Gaemers and Bax (figure 4). In addition we observed that a sample heated outside the probe to 37 °C did not show any orientation after placing it into the magnet. In contrast, the same sample did align after cooling to 4 °C and subsequently heating to 37 °C inside the probe. This observation refutes the bicel model too, because according to Ottiger and Bax (Ottiger & Bax, 1998) at 30 mg lipid/ml (molar ratio q=[DMPC]:[DHPC]=3) each bicelle should fit in its own cube of 285×285×285 Å. Consequently, bicelles still should be able to align after they are formed outside the magnet.
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Figure 4: Cryo-electron micrographs of a 4% w/v phospholipid mixture DMPC:DHPC-PEG2000-PE (q=2.9,100 mM KPi, pH 7.0, 1 molar % PEG-2000-PE) at (A) 10 °C and at (B) 53 °C. The structure of figure B is also observed at 37 °C (data not shown). It is clearly shown that the bicelles are only formed at low temperatures and that at higher temperatures a more complex structure is formed. Bars indicate 100 nm.

Although very useful, the DMPC/DHPC mixture, in which the macromolecule is oriented by steric interactions, has its disadvantages. For example, the phospholipid mixture is thermotropic, adopting only a liquid crystalline phase over a narrow range of temperatures. Second, due to the presence of hydrophobic patches the stability of the medium is affected, possibly leading to phase separation, and third, the phospholipid liquid crystalline phase is only stable over a limited pH range due to acid- and base-catalysed hydrolysis of the ester bond in DMPC and DHPC. The stability, temperature and pH range of the liquid crystalline phase can be changed as will be discussed below.

The temperature range of the liquid crystalline phase is limited between 29 and 45 °C. This range is dependent on the molar ratio (q) of DMPC and DHPC (optimum ratios fall in the range 2.9 to 3.7) and the total phospholipid concentration (3-5% w/v). For example, a 5% w/v mixture with q=3.0 forms a stable oriented phase between 33 and 45 °C at a salt concentration below 100 mM. By decreasing the [DMPC]:[DHPC] ratio the range of temperatures over which orientation is maintained is shifted to slightly higher temperatures and by lowering the total phospholipid concentration the temperature range will be extended (Losonczi & Prestegard, 1998; Ottiger & Bax, 1998). The lower temperature limit at which the phospholipids adopt a liquid crystalline phase can be lowered a few degrees by using unsaturated phospholipids like DTPC (ditridecanoyl-phophatidylcholine) instead of DMPC. A disadvantage of DTPC/DHPC mixtures is that they are very viscous at low temperatures, making it impossible to measure scalar couplings just by lowering the temperature (Ottiger & Bax, 1998). Other mixtures used at lower temperatures are the DLPC/DHPC (DLPC=dilauroyl phosphatidylcholine) and the DLPC/CHAPSO (CHAPSO=3-(cholamidopropyl) dimethylammonio-2-hydroxyl-1-propane sulfonate) mixtures. A 5% w/v solution of the latter forms a stable liquid crystalline phase above 7 °C at a DLPC:CHAPSO molar ratio of 4.2:1 (Wang et al, 1998).
An advantage of using CHAPSO instead of DHPC is that the short-chain lipid is not hydrolysable at low or high pH.

Phase separation of the oriented lamellae can be prevented by adding charged amphiphiles. Charged amphiphiles prevent aggregation of the phospholipid bilayers by the introduced electrostatic repulsion which also results in an extended temperature range of the stable liquid crystalline phase. In addition, because of the charge introduction the macromolecule studied will be oriented not only by steric interactions but by electrostatic interactions as well. Examples of charged lipids that have been used are the positively charged lipids CTAB (hexadecyl (cetyl) trimethylammonium bromide) and DMTAP (1,2-dimyristoyl-3-trimethylammonium-propane) and the negatively charged lipids SDS (sodium dodecyl sulphate), DMPG (1,2-dimyristoyl-sn-3-phosphoglycerol), DMPS (1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine) and DMPA (1,2-dimyristoyl-sn-glycero-3-phosphatidic acid) (Crowell & Macdonald, 1999;Losonczi & Prestegard, 1998;Struppe et al, 2000). The amphiphiles CTAB and SDS are added in small amounts (ratio DMPC:charged lipid of 50:1) (Losonczi & Prestegard, 1998), but the other lipids are added in higher concentrations. For example, DMPG-DMPC ratios range from 0 to 50/50 and DMTAP-DMPC ratios range from 0 to 40/60, keeping the proportion of DHPC constant relative to the sum of long-lipid molecules (e.g. DMPG+DMPC). One disadvantage of the charged amphiphiles is that the formation of a liquid crystalline phase containing charged amphiphiles is contingent upon the presence of at least 50 mM salt (Brunner et al, 2001;Crowell & Macdonald, 1999;Losonczi & Prestegard, 1998). Phase separation is also prevented by the addition of a small amount PEG2000-PE (1,2-dimyristoyl-sn-glycero-3-phospho-ethanolamine-N-[poly(ethylene glycol)2000]) (1% molar to DMPC). PEG-2000PE prevents neighbouring bilayer surfaces from approaching each other and fusing to produce insoluble aggregates. In this case, although some negative charge is introduced, the orientation of the biomolecules will be mainly a result of an obstruction effect rather than electrostatic interactions (King et al, 2000).

The third disadvantage of the DMPC/DHPC mixtures is the limited pH range. The phospholipids are subject to hydrolysis of the ester bonds in a matter of weeks or days if the pH is not carefully kept between pH 6 and pH 7. Ottiger and Bax (Ottiger & Bax, 1999) showed that substitution of the carboxy-ester bonds present in DMPC and DHPC for the ether-linkages in their alkyl analogs (i.e. 14-O-PC and 6-O-PC, respectively) prevents acid- or base-catalysed hydrolysis of these compounds. These mixtures form a stable liquid crystalline phase over the range of at least pH 1.0 to 12.5. The temperature range of the liquid crystalline phase varies with pH and is shifted to somewhat higher values compared with the conventional DMPC/DHPC mixtures. A different alternative, which is particularly useful for samples that require very acidic conditions is the DIODPC/CHAPSO system (1,2-di-o-dodecyl-sn-glycero-3-phosphocholine) presented by Cavagnero et al. (Cavagnero et al, 1999). At the optimal DIODPC/CHAPSO molar ratio of 4.3:1, this medium is stable over a wide range of temperatures (10 °C to at least 55 °C) and between pH 1 and pH 5. At pH values close to 6.5 line broadening occurs as a result of unfavourable electrostatic effects as the net charge on the liquid crystalline medium
approaches zero near neutral pH. Consequently, the DIODPC/CHAPSO medium is not suitable for studies close to pH 6.5.

The bilayers of the liquid crystalline phase orient with their normal orthogonal to the magnetic field in the different phospholipid media described above. Prosser et al. (Prosser et al., 1996) discovered that the addition of a small amount (1-10 mol% relative to DMPC) of one of the lanthanide ions (Ln$^{3+}$) Eu$^{3+}$, Er$^{3+}$, Tm$^{3+}$ and Yb$^{3+}$ causes the liquid crystalline phase to change the orientation from negative order (the normal orthogonal to the magnetic fields) to positive order (the normal parallel to the magnetic field). However, lanthanides shift and broaden NMR lines. In addition they might bind to sites of negative charge on the macromolecule studied. One way of overcoming these problems is to incorporate a phospholipid molecule that binds or chelates lanthanides (e.g. DMPE/DTPA (1,2-dimyristoyl-sn-glycero-3-phospho-ethanolamine/diethylene-triamine-pentaacetate)) (Prosser et al., 1998c). A different approach to change the alignment of the normal from perpendicular to parallel is the use of DBBPC (1-dodecanoyl-2-(4-(4-biphenyl)butanoyl)-sn-glycero-3-phospho-choline) instead of DMPC. In DBBPC one of the long aliphatic chains is replaced by a biphenyl moiety (two phenyl rings), which causes the positive magnetic susceptibility (Tan et al., 2002).

In addition to the stability of the liquid crystalline medium, the possibility to recover the macromolecule might be a selection criterion for choosing a particular medium as well. The possibility to separate the protein from the medium is of great value, especially in the case of doubly labelled protein samples. Ottiger and Bax (Ottiger & Bax, 1998) proposed two procedures to separate the protein from the phospholipid mixtures.

1. Shake the phospholipids mixture with 5 volume fractions of a 2:1 (v/v) chloroform methanol solution. Dialysis of the protein containing aqueous fraction can be used to remove to methanol and residual DHPC.

2. Dilute the mixture with a 20 fold amount of water. Centrifuge at 4°C and concentrate the sample. Dialysis can be used to remove the DHPC. Small amounts of residual DMPC can then be spun down.

**Sample preparation**

Different methods are described in the literature for the preparation of phospholipid mixtures (Losonczi & Prestegard, 1998; Ottiger & Bax, 1998). In our laboratory we use a method which is a combination of previously published methods. First, DMPC and DHPC stock solutions of 20% w/v are prepared by carefully weighing the appropriate amount of phospholipids and dissolving it in buffer (these solutions can be stored frozen, preferably at -80°C). Both solutions have to go through several freeze-thaw cycles followed by vortexing until all the DMPC and DHPC is dissolved. Second, a fraction of the DHPC stock solution is added to a vial containing a predetermined amount of the DMPC stock solution, sufficient to yield the desired molar ratio [DMPC]:[DHPC] (Losonczi & Prestegard, 1998). We advise to add a small amount (1% molar to DMPC) of
a stock solution PEG2000-PE to prevent phase separation (King et al, 2000). The mixture is briefly vortexed, heated to 40 °C for several minutes and cooled on ice to 0 °C. This cycle is repeated 3 times to ensure sample homogeneity (Ottiger & Bax, 1998). Eventually, when the sample is homogeneous the sample should be clear, but after placing it under a hot tap, it should become white (the transition starts at about 25 °C). Finally the protein, dissolved in the appropriate buffer, can be added to the sample. Because macroscopic phase separation might occur around the transition temperature of DMPC, the sample has to be kept on ice until use and placed in a pre-heated probe. In addition phase separation can be minimised by quickly cooling the sample tube in ice-water after use. In case phase separation occurs the homogeneity of the sample can be restored by cooling to 4 °C, followed by vortexing.

**Surfactant / n-alkyl alcohol mixtures**

In addition to the phospholipid mixtures, a range of other liquid crystalline media has been demonstrated to achieve a useful weak degree of macromolecular alignment. One of these media published soon after the introduction of the “bicelles”, is the liquid crystalline phase consisting of a 2-5% (w/w) aqueous solution of cetylpyridinium chloride (CPCl) and n-hexanol (Prosser et al, 1998a). Equal weight fractions of CPCl and hexanol reveal a stable liquid crystalline phase between 0 and 70 °C. However, salt concentrations of at least 200 mM NaCl are necessary.

An alternative for the CPCl/hexanol medium is the Cetylpyridiniumbromide (CPBr) /hexanol liquid crystal published by Barrientos et al. (Barrientos et al, 2000) (figure 5). In contrast to the choride system, only small amounts of salt (25-30 mM NaBr) are necessary to stabilize the oriented phase. For example, mixtures containing 4-6.5 % w/v CPBr-hexanol (ratio CPBr:hexanol 1:1.33 (w/w)) and 25-30 mM NaBr are stable in the range 15-60 °C and at a wide range of pH values (pH 2-8) and buffer types.

![Molecular structure of (A) CPBr and (B) hexanol.](image)

Figure 5: Molecular structure of (A) CPBr and (B) hexanol.

The exact morphology of the liquid crystalline medium is still under debate. Initially, an α-lamellar phase, the so called Helfrich phase (Helfrich, 1978), was expected. Recently, Barrientos et al. (Barrientos et al, 2002) proposed a different model, based on electron microscopy images, consisting of closely packed elongated wormlike structures that are 5.5 nm wide and ranging up to 1 μm in length. On the contrary,
Gaemers and Bax (Gaemers & Bax, 2001) exclude the possibility of wormlike morphologies, which they conclude from tracer self-diffusion experiments.

Irrespective of the morphology of the liquid crystalline phase, the medium orients with the symmetry axis of the lamellae/worms parallel to the magnetic field (figure 3B). This orientation is dictated by the magnetic anisotropy of the pyridinium ring. Because this pyridinium ring is positively charged at neutral pH, the mechanism of orientation is mostly electrostatic in the case of transiently interacting proteins (Burnell & de Lange, 2003).

Another liquid crystalline medium suitable for partially aligning macromolecules is the quite robust and easy to make alkyl poly(ethyleneglycol)/n-alcohol mixture as described by Rückert and Otting (Rückert & Otting, 2000). The different alkyl poly(ethyleneglycol) mixtures are denoted as CmEn, where m is the number of carbons in the linear alkyl chain and n is the number of the glycol units in the poly(ethylene glycol) moiety (e.g. C12E6, C12E5 and C8E5). Molar ratios of CmEn to n-alkyl alcohol are in the range 0.64-0.96 and weight percentages of CmEn are between 3 and 11% (Liu & Ding, 2003).

![Figure 6: Temperature range of stable lamellar phases composed of CmEn/alcohol/H2O and glucopone/alcohol/H2O. The systems are identified by the surfactant/H2O ratio in wt % taking into account the presence of D2O, but not the alcohol. In the glucopone/n-hexanol mixture the n-hexanol concentration is given on a total solution weight basis for the pure liquid crystal. The r-value denotes the molar ratio surfactant to alcohol.](image)

The alkyl poly(ethyleneglycol)/n-alcohol mixtures form a lyotropic liquid crystalline phase that orients with the normal perpendicular to the magnetic field. In this so called Lα phase, the hydrophobic n-alkyl chains aggregate into planar bilayers with the hydrophilic headgroups directed into the water phase. A superstructure of concentric cylinders is proposed (figure 3C), but could neither be confirmed nor disproven on the basis of tracer diffusion data (Gaemers & Bax, 2001). The temperature range of the liquid crystalline phase depends on the n-alkyl alcohol concentration. In fact, the function of the
$n$-alkyl alcohol is two-fold: first, the addition of $n$-alkyl alcohol (octanol or hexanol) lowers the temperature range of stability for the liquid crystalline phase, and second, it makes the Lα phase accessible even at low surfactant concentrations (figure 6).

$CmEn/n$-alcohol mixtures are insensitive to pH, fairly insensitive to salt and have a low affinity for biological macromolecules because the ethylene glycol unit is noncharged. Nevertheless, we observed that the proteins IIB$^{mtl}$ and IIB$^{chb}$ bind to the medium. By decreasing the concentration $CmEn$ the interaction between the proteins and medium reduced, however the interaction remained too strong. Others reported binding to the $CmEn/n$-alcohol liquid crystals too (Ohnishi & Shortle, 2003). Because the mixture has a low surface charge density, the alignment of the macromolecules studied is primarily caused by an obstruction effect (Gaemers & Bax, 2001).

The proteins we studied (IIB$^{mtl}$ and IIB$^{chb}$) denatured in the $n$-alkyl-poly(ethylene glycol)/$n$-alkyl alcohol mixtures, making recovery of the protein superfluous. Nevertheless, it is possible, however hard, to recover the protein by extensive dialysis followed by cationic exchange chromatography (Ruckert & Otting, 2000).

![Figure 7: $^2$H spectra of 7% w/v CPBr/hexanol (1:1.33 w/w) in 10 mM Tris-acetate pH 6.2, 25 mM NaBr, 5% glycerol and 10% D$_2$O. Spectra are recorded at 20 °C after (A) 20 min, (B) 1 hour, (C) 4 hours and (D) 1 day equilibration.](image)

Sample preparation CPBr/hexanol mixtures

Samples containing CPBr and hexanol can be prepared following the procedure described by Barrientos et al. (Barrientos et al, 2000). First a 7 % (w/v) stock solution of the liquid crystalline medium (CPBr:hexanol = 1:1.33, w/w) has to be prepared by mixing the desired amounts of CPBr, hexanol, buffer, D$_2$O and aliquots of a stock solution of 1M NaBr. HBr and NaOH can be used to make minor pH adjustments. Second, the mixture is vortexed and heated to 70 °C until the sample is clear. After clearance the sample is left to cool to room temperature and centrifuged for two minutes at 6000 g. Finally the stock solution can be mixed with a protein solution. The alignment of the CPBr liquid crystal relative to the magnetic field is a very slow process (Gaemers & Bax, 2001). We observed that the equilibration is dependent on the buffer and that it might even take a day to
equilibrate (figure 7). Samples can be pre-aligned underneath the magnet on the probe connector plate. After aligning the samples stay aligned on the bench for 1-2 more days (Barrientos et al., 2000).

Sample preparation n-alkyl-poly(ethylene glycol)/n-alkyl alcohol mixtures

$n$-Alkyl-poly(ethylene glycol)/$n$-alkyl alcohol mixtures can be prepared easily by dissolving the desired amount of $C_mE_n$ in the appropriate buffer which already contains the protein. In the next step the $n$-alkyl alcohol has to be added followed by vigorous vortexing. During vortexing the sample turns from biphasic to opalescent and transparent upon crossing the $\alpha$ phase boundary. The air bubbles can be removed from the final viscous phase by centrifugation (table top centrifuge; 30 s, 10,000 g) (Ruckert & Otting, 2000). The final sample has to be transferred carefully to an NMR tube, to avoid new air bubbles. From our experiments we can conclude (data not shown) that the liquid crystalline medium remains stable for at least two months, even when the protein binds to the medium.

We observed that it is also possible to add the $C_mE_n$ and the alcohol to an NMR tube which already contains a protein solution. After adding the compounds, the sample should be vortexed and left on the desk for several hours to get rid of the air bubbles.

Rod-shaped virusses

Probably the most widely used liquid crystalline medium is the filamentous bacteriophage Pf1. It owes its popularity, in part, to being commercially available, but it is also a robust medium and the degree of alignment of the dissolved macromolecule can be easily tuned by simply changing the phage concentration, thereby modulating the size of the dipolar couplings. In addition, the Pf1 phage is already fully aligned at 300 MHz, the protein is easily recovered and the alignment is stable over a reasonably large range of temperatures (5 °C to at least 45 °C).

The bacteriophage Pf1 consists of a 7,349-nucleotide circular single strand DNA genome with approximately one coat protein per nucleotide. The coat proteins, arranged in an $\alpha$-helical structure, pack around the DNA and are the source of the large anisotropy of the phage’s magnetic susceptibility, causing them to align with the long axis of the phage aligning parallel to the magnetic field (figure 3D) (Hansen et al., 1998). The phage particles themselves are ~60 Å in diameter and ~20,000 Å long. They have a negatively charged surface (pl=4.0) (Zimmermann et al., 1986) and a molecular weight of ~40 MDa. Thus 30 mg of Pf1 phage (~4.5 x 10¹⁴ particles) expose ~17 m² of surface area oriented parallel to the magnetic field (Hansen et al., 2000). This high degree of oriented surface combined with the low degree of alignment needed for NMR solution studies (<10⁻³), makes it possible to use only low concentrations of phage to induce sufficient alignment (1-50 mg/ml) (figure 8).

The structural details suggest that it is safe to consider a Pf1 molecule as an infinitely long uniformly negatively charged rod (at pH 7.4 the approximate linear charge
density is 10 electron charges/nm) (Vyas et al., 2002; Zweckstetter & Bax, 2001). Because the phage is charged, the orientation of the macromolecule depends on both the steric repulsion and the electrostatic contribution. For example, in case of a prolate macromolecule the excluded volume is minimized when the long axis is parallel to the director, whereas in the case of an oblate molecule minimisation is reached when the orientation is perpendicular to the director. Electrostatic contributions take the lowest values for orientations that allow positive charge groups to lie close to negative charge groups and vice versa (Ferrarini, 2003). One important feature of the phages is the possibility to change the orientation tensor of the macromolecule. For example, by increasing the ionic strength, the electrostatic interaction will weaken, but the steric effect is unchanged, thereby modulating the alignment.

Figure 8: Quadrupolar splittings of the $^2$H NMR signal of a 10 mM Tris pH 8.0, 10% D$_2$O sample containing different concentrations of Pf1. Spectra are recorded at 25°C.

Recommended pH values for phage solutions are between pH 6.5 and 8. Below pH 6 the phages will aggregate, resulting in less alignment of the phage (Hansen et al., 1998). Because the phages are negatively charged at physiological pH, Pf1 filamentous phage is especially well suited for measuring dipolar interactions in negatively charged proteins, and in RNA and DNA oligomers, which are also negatively charged at physiological pH (Hansen et al., 2000). If the macromolecule and phage interact too strongly, the electrostatic interactions can be weakened by increasing the ionic strength (figure 9) or by reducing the total Pf1 concentration. However, in both cases the alignment will be reduced. In addition, the salt concentration influences the nematic phase boundary. Below the boundary the mixture is paramagnetic and cooperative alignment depends on the strength of the field. Above 16 mg/ml the phage suspension is fully nematic up to 600 mM NaCl, and alignment might be expected to be independent of the magnetic field.
strength. Cai et al. (Cai et al., 2003) showed that ionic concentrations 0.5 mM NaCl might be necessary to abolish the interaction between the Pf1 phage and the protein studied. Apart from the Pf1 and salt concentration, alignment is also dependent on the temperature, although the alignment decreases only a few percent by increasing the temperature from 7 to 25 °C.

Proteins can be recovered from Pf1 solutions by centrifugation at 436000 g for 1 h in a Beckman TL-100 tabletop centrifuge. Some salt must be present in the solution, because the phage will pellet poorly from a solution without salt (Hansen et al., 2000; Zweckstetter & Bax, 2001). In spite of the electrostatic interactions between the protein studied and Pf1 (figure 9a), we were able to recover more than 90% of the protein using the method described.

The same procedure can be used to change the buffer of a Pf1 solution. After centrifugation the phage can be dissolved in the appropriate buffer (containing salt) and centrifuged again. Phage recovery will be 50-70%.

Clore et al. (Clore et al., 1998) demonstrated the use of filamentous bacteriophages fd and tobacco mosaic virus (TMV) as an orienting medium for macromolecules. Just like Pf1 phages, these two media undergo a magnetic field induced transition from an isotropic to a nematic phase at moderate concentrations. Both media are stable between 5 to 60 °C and precipitation occurs when the pH of the solution approaches the pI (4.2 for fd and 3 for TMV). Fd closely resembles Pf1 in morphology. The only gross morphological distinction between Pf1 and fd is their length: Pf1 is approximately twice as long as fd (Barrientos et al., 2001). Because the degree of alignment, and hence the size of the dipolar couplings is related to the length of the particle, the nematic threshold concentration for Pf1 is approximately two-fold lower than for fd. Whereas the

![Figure 9: HSQC of 13C- and 15N enriched IIB mtl in 20 mM Tris pH 7, 1 mM EDTA, 1 mM DTT, 1 mM NaN3, 10% D2O, 17 mg/ml Pf1 and (A) 0 mM NaCl and (B) 600 mM NaCl.](image)
Media used for orienting biological macromolecules

Surface electrostatic properties are quite similar, it is expected that for proteins that have an electrostatic interaction with Pf1 and fd, working with Pf1 is preferred because lower Pf1 concentrations (mg/ml) can induce the same degree of alignment at a smaller cost in increased relaxation (Zweckstetter & Bax, 2001). A problem of TMV as an orienting medium is its tendency for phase separation. This phase separation can be reversed if a small amount (0.1 % w/v) of CTAB is added (Brunner, 2001).

Sample preparation

Pf1 phage suspensions of up to 50 mg/ml can be purchased from ASLA. Add the desired amount (often 16-27 mg/ml) of phage solution to the NMR sample and mix it by intensive pipetting followed by 1 minute of centrifugation at 10,000 g on a table centrifuge to remove air bubbles. Sample concentrations can be verified by absorbance at 270 nm using an extinction coefficient $\varepsilon = 2.25 \text{ cm}^{-1} \text{ mg}^{-1} \text{ ml}$. Due to the high viscosity of the phage solution, errors in Pf1 concentrations are estimated at ~5% (Zweckstetter & Bax, 2001). The final sample is still very viscous, but by attaching a Teflon tube (1.5 - 3 mm inner diameter) to the 1 ml tip for the automatic pipette the sample can be transferred to the NMR tube. It is important to release the sample carefully at the bottom of the NMR tube to avoid new air bubbles. A slow decrease in alignment, on the order of 1% per month, is commonly observed. It may reflect a slow decay of the Pf1 structural integrity (Bax, 2003).

Purple membrane fragments

Purple membranes (PM) are naturally occurring two-dimensional crystals and are derived from the *Halobacterium salinarium*. They form large disks consisting primarily of a bacteriorhodopsin monomer and seven or eight mostly negatively charged residual lipids. Per monomer the magnetic susceptibility anisotropy is determined as $1.2 \times 10^{-2} \text{ J/mol/T}^2$. Because a typical purple membrane sheet contains about $4 \times 10^4$ such units, the energy of the magnetic alignment for a whole purple membrane crystal ($\chi_{pm}B^2/2$) is more than 1 order of magnitude larger than the thermal energy at room temperature for a typical magnetic field (B>10 Tesla) in high field spectrometers. This results in almost full alignment of individual particles at field strengths > 10 Tesla with their normal parallel to the magnetic field (figure 3E). The alignment is temperature independent over a wide range and scaleable by the addition of more PM fragments. Unlike the liquid crystalline media described above, the biological PM fragments have no critical lower concentration threshold to order in a magnetic field (Koenig et al, 1999; Sass et al, 1999).

PM fragments are highly negatively charged and consequently induce orientation of biomolecules mainly via electrostatic interactions. Because of the highly negative charge of the PM fragments, the PM system is most likely suitable for proteins at pH values above their pI and the negatively charged nucleic acids (Sass et al, 1999). The electrostatic interactions and thus the orientation of the macromolecule can be modulated by varying the concentration of PM fragments or by addition of salt. However, PM
fragments are very sensitive to the ionic strength and at salt (NaCl or KCl) concentrations greater than 70 mM the PM suspensions undergo a transition from a fluid to a highly viscous state. For MgCl₂ this transition is already observed at salt concentrations <5 mM. The mechanism underlying the salt-induced gelation is the screening of repulsive electrostatic forces between the negatively charged PM fragments. Consequently, van der Waals interactions overcome the electrostatic repulsion and aggregation of the membranes will take place. When kept at low ionic strength samples remain stable for a few months over a wide temperature (until 69 °C) and pH (2.5 – 10) range (Koenig et al., 1999; Sass et al., 1999). Typically concentrations of PM fragments used for residual dipolar coupling measurements are 1-4 mg/ml.

Because of the high negative charge of the PM fragments, the PM system is most likely suitable for proteins at pH values above their pI and negatively charged nucleic acids (Sass et al., 1999).

Sample preparation

Purple membrane fragments can be isolated and purified from *Halobacterium salinarium* cells as described by Oesterhelt and Stoeckenius (Oesterhelt & Stoeckenius, 1974) and Bauer et al. (Bauer et al., 1976). Alternatively they can be purchased from Munich Innovative Biomaterials GmbH (MIB). Samples are prepared by simply titrating suitable amounts of purple membrane into the protein solution. In view of the above, salt (>70 mM NaCl or KCl, >5 mM MgCl₂) has to be added very carefully by pipetting gently a droplet of a concentrated salt solution at the meniscus of the air/water interface of the PM suspension. Immediately afterwards, this sample must be placed in the magnet to avoid any further turbulence in the suspension, while the salt diffuses into the sample. If the transition is induced outside the magnet, the PM fragments will not be able to align after placing the sample in the magnet. In the latter case, the magnetic torque exerted on the PM fragments is too small to overcome the frictional forces in the viscous sample (Koenig et al., 1999; Sass et al., 1999).

Strained polyacrylamide gels

Another system that is useful and practical for inducing alignment makes use of anisotropic compression or stretching of polyacrylamide gels. This method, often referred to as SAG (strain induced alignment in gel), was independently introduced by Sass et al (Sass et al., 2000) and Tycko et al (Tycko et al., 2000). This system is described as inert and stable over a wide ranges of temperature (at least 5-45 °C), pH values (at least 2.0-8.5) and ionic strength (0 mM to at least 200 mM) (Ishii et al., 2001). In addition, protein is simply recovered by immersing the gel (preferably minced) in a small volume of distilled water. After several days, the supernatant can be concentrated.

Compression of a gel is achieved by using a plunger for pushing onto a gel which has a slightly smaller diameter (0.5 mm) than the inner diameter of the NMR tube. As a
consequence the cavities in the gel will be oblate with their normal in the direction of the tube. Stretching usually refers to radial compression and is accomplished by squeezing a larger diameter gel into a regular NMR tube, resulting in prolate cavities (figures 3F and 3G). The alignment in these electrically neutral polyacrylamide gels is dominated by steric interactions. So, the macromolecule aligns with its long axis perpendicular to the magnetic field in a compressed gel and parallel to the field in a stretched gel. Embedding of positive (Ulmer et al., 2003) or negative (Meier et al., 2002; Ulmer et al., 2003) charges into the acrylamide gels causes alignment by electrostatic interactions, which leads to markedly different orientation tensors.

Although polyacrylamide gels are widely used to align macromolecules, they might disturb the $^1$H-$^{15}$N spectrum of the macromolecule studied by introducing several peaks between 114-116 ppm $^{15}$N and 7-7.8 ppm $^1$H (figure 10). A second disadvantage of SAG is that it can inhibit the rotational diffusion rate of the dissolved macromolecules, thereby increasing resonance line widths and decreasing NMR sensitivity. This effect can be minimized by decreasing the gel density, and it therefore is desirable to use the lowest possible gel density and largest possible compression factor (Bax, 2003; Sass et al., 2000). However, a lower density results in less alignment of the dissolved macromolecule. In these cases it is desirable to stretch the gel. Although compression is technically simpler than stretching, stretching can provide a larger net alignment as a result of the asymmetrical distribution of the dipolar couplings around zero, reflecting the function $3\cos^2\theta-1$. Stretching causes the long axis of the molecules studied to align parallel instead of orthogonal to the magnetic field, which approximately doubles the range of dipolar couplings for a given gel density and aspect ratio (Ackerman & Shortle, 2002; Bax, 2003).

Figure 10: Small region of the $^1$H-$^{15}$N HSQC spectrum of a 7.3% w/v polyacrylamide gel, with a compression factor 0.75.
Sample preparation

Here we present one method for compressing polyacrylamide gels based on the procedure described by Sass et al. (Sass et al., 2000). Dependent of the desired pore radii, it might be necessary to try different acrylamide and methylenebisacrylamide cross linker concentrations (Ishii et al., 2001). Concentrations of the gel ($C_A$) and the cross-link density ($F_{CL}$) range from 5.1-10.2 % w/v and 2.9-5.0% w/v, respectively. $C_A$ is the acrylamide concentration, $F_{CL}$ is $C_B/(C_A+C_B)$ and $C_B$ is the N,N′-methylenebis-acrylamide concentration, which ranges from 0.23 to 0.54% w/v. For compressed gels it is important to make sure that the total volume of the original gel is nearly identical to the total volume of the compressed gel. We observed that in cases that the volume of the strained gel was smaller than the original volume, the NMR tube might break.

An acrylamide solution is prepared by diluting the commercially available 30% acrylamide/bis solution (29.2% w/v acrylamide and 0.78% w/v N,N′-methylenebisacrylamide) with demineralised water until the desired concentration (e.g. 7.3% w/v). Then add 0.1 % w/v ammoniumpersulphate (APS) and 0.5 % w/v N,N,N′,N′-tetramethylene diamine (TEMED). Vortex the suspension vigorously and cast the gels in glass tubes (Ishii et al., 2001) which are closed on one end by parafilm and have an inner diameter ~0.5 mm smaller than the inner diameter of the NMR sample tube. After polymerisation the gel can be pushed gently out of the tube with a plunger. Next, the gels are washed in water for several hours and dried overnight on a smooth plastic support (e.g. household foil wrapped around a smooth surface) in a drying oven at 37 °C. Once dried, the gel can be placed in an NMR tube and the desired solution, containing the macromolecule, can be added and the gel will start to swell. Pressure from the plunger can be applied when the gel has reached a diameter slightly smaller than the inner diameter of the NMR tube. Without a small cleft it is very hard to compress the gel. On the contrary, a cleft that is too big will result in coiling of the gel. Compression factors often lie between 0.75 and 0.85 (length of sample after compression/initial length after polymerisation).

The method described above can also be used for stretched gels. However, the gels have to be casted in tubes with a larger inner diameter (e.g. 6 mm) than the NMR sample tube and during reswelling of the dried gel no vertical pressure has to be applied. For the last procedure it is necessary to coat the NMR tube with dimethyldichlorosilane (Sass et al., 2000) or propyltrimethoxysilane (Chou et al, 2001). Drying of the sample is superfluous when the commercially available apparatus developed by Chou et al.(Chou et al., 2001) is used to insert the gel into the NMR tube (figure 11). In the last case, protein is added before polymerisation (http://www.newera-nmr.com/).

The preparation of charged polyacrylamide gels is performed in analogy to the method for uncharged polyacrylamide gels. Ulmer et al (Ulmer et al, 2003) replaced 5% acrylamide by an equimolar amount of 2-acrylamide-2-methyl-1-propanesulfonic acid (AMPS) or 10 times the equimolar amount of diallyldimethylammonium chloride (DADMAC) to introduce negative or positive charge, respectively. Meier et al (Meier et al, 2002) replaced a part (max 40%) of the commercially available stock solution (29.2% w/v acrylamide and 0.78% w/v bis) by the stock solution acrylic acid 29.2% w/v /
bisacrylamide 0.78% w/v (pH 4.25). In the latter case we would recommend to start with a low concentration of acrylic acid (e.g 20%), because higher acrylic acid concentrations make the gels more fragile after washing several times for a few hours with a ~100-1000 fold excess of ultra pure water (Milli-Q).

Figure 11: Apparatus presented by Chou et al. (2001) for stretching the gel and inserting it in the open-ended NMR tube.

**Polymer-stabilized liquid crystals**

By embedding Pf1 (Trempe et al., 2002) or purple membranes (Sass et al., 2000) in a polyacrylamide gel the orientation of the embedded particles can be made permanent when the polymerisation is carried out within the magnet. This type of orienting media is called a polymer-stabilised liquid crystal (PSLC).

An advantage of such media is that the orientation of the liquid crystal director with respect to the magnetic field can be varied. This makes it possible to measure J and J+D couplings in one sample without changing the temperature. Because the residual dipolar couplings are scaled by the second order Legendre polynomial:

\[ P_2(\phi) = \frac{3 \cos^2 \phi - 1}{2} \]

in which \( \phi \) is the angle between the liquid crystal director and the magnetic field, the RDCs in samples with the particles aligned at the magic angle (\( \phi = 54.7^\circ \)) are close to zero (Trempe et al., 2002).

Riley et al (Riley et al., 2002) introduced an alternative polymer matrix for embedding Pf1: the commercially available surfactant Pluronic F-127. Pluronic F-127 consists of poly(propylene oxide) and poly(ethylene oxide) and forms a viscous gel at concentrations > 20% w/v at room temperature. At low temperatures the suspension is a flowing liquid. Because the viscosity transition is reversible, it is easy to recover the macromolecular solution by cooling the sample to 4 °C followed by centrifugation.
Sample preparation

Polymer-stabilized liquid crystals are prepared in a similar way as a polyacrylamide gel with the difference that Pf1 phages or purple membranes are added to the solution before polymerisation. The polymerisation has to take place inside the bore of the magnet (Sass et al., 2000; Trempe et al., 2002).

Trempe et al. (Trempe et al., 2002) describe a method in which complete polymerisation will take about 20 minutes, making it possible to vortex and centrifuge the sample before polymerisation. After dilution of a stock solution of 29% w/v acrylamide / 1% w/v N,N'-methylenebisacrylamide with a H2O/D2O/protein solution they start the polymerisation by adding 0.1% APS and 0.1% TEMED. Next, the sample is vortexed and centrifuged after addition of Pf1 or purple membranes. Finally the sample is transferred to an NMR tube and placed in the magnet to let it polymerize (Trempe et al., 2002).

Cellulose crystallites

Fleming et al. (Fleming et al., 2000) introduced the aqueous suspensions of cellulose crystallites as a new liquid crystalline medium for the measurement of residual dipolar couplings in soluble macromolecules. These crystallites are typically 200±70 nm long and 10 nm wide and are stabilised by negatively charged sulphate groups (OSO$_3^-$) on their surface (Dong & Gray, 1997). The cellulose crystallites spontaneously form an ordered chiral nematic liquid crystalline phase when the suspension concentration is higher than the critical concentration of 4.5% w/v cellulose. This chiral nematic phase consists of stacked planes of molecules aligned along a director in which the orientation of each director is rotated about the perpendicular axis from one plane to the next (figure 3H). Because the particles possess a large negative diamagnetic anisotropy, they align with their long axis perpendicular to the applied magnetic field. As a result, the chiral nematic structure is not disrupted by the magnetic field, but rather lines up the chiral nematic axis along the direction of the field. This alignment process occurs over a period of hours to days (Fleming et al., 2001).

The medium is expected to resist wide pH variations, but the exact stability against different experimental conditions is still unknown. Also the mechanisms of orientation of the macromolecules still remains to be characterized, but should partially be electrostatic (Burnell & de Lange, 2003). The addition of salt to destabilize unwanted electrostatic interactions is often unnecessary, even in the case of highly basic proteins (e.g. intimin; pI=8.9) (Fleming et al., 2000). For this reason, cellulose crystallites seem to be particularly useful for highly charged molecules that interact with other orienting media (de Alba & Tjandra, 2002).

Sample preparation

A suspension of cellulose microcrystallites can be prepared in large amounts from ground Whatman No. 1 filter paper powder as described by Dong et al. (Dong et al,
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1996; Dong & Gray, 1997). They mix the paper (< 20 mesh) with sulphuric acid (64% w/v) (ratio filter:paper 1:8.75 (g/ml)) and stir it at 45 °C for 1 hour. After hydrolysis the crystallites are dispersed by an ultrasound treatment (5 min) with a sonifier cell disrupter 350 watts (Branson Sonic Power Co.). During the dispersion the sample has to be kept in an ice bath in a plastic container. Next, the suspension is deionized by mixing it with a mixed-bed ion-exchange resin (e.g. Rexyn I-300 H-OH) after which the sample is purified by filtration through a 0.45 μm membrane followed by centrifugation. The acid is removed by extensive dialysis against pure water. Finally water is allowed to evaporate, which results in a lower anisotropic and an upper isotropic phase. When the lower anisotropic phase makes up about 30% of the biphasis suspension volume (critical concentration >4.5%) the phases can be separated. The isotropic phase can be concentrated further until about 30% of the suspension is isotropic, after which the phases can be separated again. The anisotropic fractions have to be concentrated further by evaporation (Fleming et al, 2000) to a maximum of 15% w/v. At higher concentrations the suspensions are too viscous. Finally the cellulose suspensions can be added to either a protein solution or to freeze dried protein.

Mineral liquid crystals

Desvaux et al., (Desvaux et al, 2001) demonstrated the use of the mineral liquid crystal (MLC) V₂O₅ (vanadium pentoxide) for the measurement of residual dipolar couplings. With V₂O₅ concentrations between 0.13M and 0.2M a stable nematic phase is formed, which easily orients in a magnetic field. At concentrations higher than 0.2 M the suspension becomes too viscous, which prevents orientation in a magnetic field. The nematic phase contains ribbons with a negative linear electrical charge of about 1e/ Å at pH 2 and with typical dimensions of 1 nm thick, about 20 nm wide and 600 nm long (Camerel et al, 2003). The mechanism responsible for the orientation arises from the strong magnetic anisotropy, aligning the ribbons parallel to the magnetic field (figure 3I). The orientation of the biomolecule is mainly caused by electrostatic interactions, possibly including hydrogen bonds.

Advantages of this medium are: i) only very small amounts of mineral liquid materials are needed (~2-3% w/w); ii) it is stable on a very long timescale (a few years) and over a wide range of temperatures (the liquid-state temperature of the solvent); and finally, iii) the dissolved biomolecule can simply be recovered by flocculation of the mineral colloid by increasing the ionic strength of the medium. After centrifugation, the biomolecule can be extracted from the supernatant by methods such as chromatography or dialysis. One disadvantage of this medium is the limited pH range (pH 1-3) in which it is stable (Desvaux et al, 2001).
One other mineral liquid crystal used for structural NMR studies is $\text{H}_3\text{Sb}_3\text{P}_2\text{O}_{14}$. At an overall mineral volume fraction, $\phi^1$, between 0.75 and 1.78, the suspension will form fluid birefringent suspensions, consisting of a lyotropic liquid-crystalline lamellar phase comprising an aqueous dispersion of planar solid-like sheets (1nm thick and 300 nm in diameter) (Gabriel & Davidson, 2003). These sheets align in a magnetic field with their normal parallel to the magnetic field and are stable in the whole temperature range useful for biomolecules (4-50 °C). After addition of a dilute base (<0.3 M) with a bulky counter ion to avoid flocculation (e.g. tetramethylammonium hydroxide or tetra(tributyl)ammonium hydroxide), the medium is stable at a wide pH range (pH 2.5-9.5). Salt concentration must be < 100 mM to avoid flocculation of the gels (Gabriel et al, 2001).

Important to note is the fact that $\text{V}_2\text{O}_5$ and $\text{H}_3\text{Sb}_3\text{P}_2\text{O}_{14}$ might change the conformational equilibrium in flexible domains of the molecule studied. This result is not causing a problem for the structure determination of large structured biomolecules, because it is likely that any conformational exchange affecting a flexible part in the molecule cannot largely change the alignment tensor. However, the alignment tensor of small molecules (e.g. oligosaccharides) might change. From a physical point of view, this effect is not surprising since the molecular alignment results from interactions between (Berthault et al, 2003).

Sample preparation $\text{V}_2\text{O}_5$

A pathway for synthesizing $\text{V}_2\text{O}_5$ solutions for NMR experiments is described by Desvaux et al. (Desvaux et al, 2001). These authors passed 6.5 ml of a 1M Sodium metavanadate dissolved in D$_2$O through a column (diameter 1.1 cm, height 19.5 cm) filled with 6.15 g proton-exchanging resin (Dowex 50 W-X2, 50-100 mesh) and subsequently eluted with 40 ml D$_2$O. Once the eluent became coloured, they started to collect in 2 ml fractions. After 24 hour the fractions with homogeneous, viscous, birefringent dark red gels were diluted with D$_2$O, until the concentration of 0.15 M $\text{V}_2\text{O}_5$ (2.34% w/w) was reached. Before use, the suspensions had to age for one month. Finally, the macromolecule studied can be dissolved in the obtained $\text{V}_2\text{O}_5$ solution. Once the ribbons are aligned, the samples stay aligned outside the magnet for several days (Commeinhes et al, 1997).

Sample preparation $\text{K}_3\text{Sb}_3\text{P}_2\text{O}_{14}$

A detailed procedure for a three-step synthesis is given in Gabriel et al. (Gabriel et al, 2001) as supplementary information. In short the three steps are: 1) high temperature solid-state synthesis of $\text{K}_3\text{Sb}_3\text{P}_2\text{O}_{14}$; 2) exchange of the potassium cations by protons in

---

1 The overall mineral volume fraction, $\phi = \frac{M c}{\rho}$; where $c$ is the concentration in mol/l, $M$ is the molecular mass in g/mol and $\rho = 2.4$ g/cm is the density of $\text{H}_3\text{Sb}_3\text{P}_2\text{O}_{14}$. If $0.75<\phi<1.78$ fluid birefringent suspensions are formed, below $\phi = 0.75$ suspensions are biphasic and above 1.78 the mixtures form birefringent gels.
Media used for orienting biological macromolecules

concentrated nitric acid, and finally, 3) a thorough rinsing and dialysis of the solid obtained to remove remaining nitrate ions. This procedure leads to homogeneous, transparent and colourless suspensions. The viscoelastic properties of these suspensions depend on the overall mineral volume fraction.

Lanthanide-binding tags

If none of the media described above appear to be inert and stable under the required conditions, there is one more alternative for aligning the biomolecules in a magnetic field. This method, which makes use of a lanthanide-binding tag (LBT), was recently presented by Wohnert et al (Wohnert et al, 2003b).

The LBTs, which were developed by the group of Imperiali (Franz et al, 2003; Nitz et al, 2003), are protein fusion partners consisting of encoded amino acids (LBT = DYNKDGWYEELE) that bind lanthanide ions with high affinity. After incorporation of the LBT in the protein studied, the biomolecules are able to achieve significant alignment in a magnetic field, without the need for an external alignment medium but in the presence of stoichiometric amounts of lanthanide ion (e.g. Tb$^{3+}$, Lu$^{3+}$, Tm$^{3+}$, Dy$^{3+}$).

On the webpage of his group Griesinger presents another lanthanide binding tag (http://medusa.nmr.mpibpc.mpg.de). They introduce lanthanide ions by a chemically synthesised and modified EDTA compound. The EDTA compound can coordinate a lanthanide ion and binds to a cysteine residue through a disulfide bond. Of course the cysteine residue itself must not be involved in a disulfide bond that is important for the structure of the protein. In addition, the cysteine must not be located in a part of the molecule where the EDTA-tag might change the structure of the molecule.

Sample preparation

A detailed method for the preparation of LBT-containing plasmids, the transformation of the LBT-plasmids into BL21-Gold(DE3) competent cells and the subsequent protein expression and purification is described by Franz et al. and Nitz et al. (Franz et al, 2003; Nitz et al, 2003). For preparation methods for the lanthanide binding EDTA tag we advise the reader to contact the group of Professor C. Griesinger.

Conclusions

We have described many different media that can be used for orienting biological molecules, none of which is universally applicable. While looking for the most suitable medium, it is important to know the pH and salt concentration of the sample, the temperature at which the experiment will be done, the charge (positive or negative) of the macromolecule studied and finally the desired orientation (perpendicular or parallel to the magnetic field). In Table 1 a schematic overview is given of the properties of the different media that are discussed in this review.

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Materials and methods

Cryo-electron microscopy

One phospholipid mixture (4% w/v DMPC:DHPC-PEG2000-PE, q=2.9, 100 mM KPi, pH 7.0, 1 molar % PEG-2000-PE) was prepared and subsequently divided into two samples. Both samples were pipetted into a 1.5 ml Eppendorf tube. Before use, one phospholipid mixture was placed in a waterbath of 10 °C and one in a waterbath of 53 °C. Samples were left in the baths for 15 minutes to equilibrate. After equilibration a small droplet of the mixture was placed on a holey carbon coated grid and the excess of liquid was blotted away with filter paper. To avoid evaporation, the grid was placed in a box containing 100% relative humidity. The samples were vitrified by plunging them into liquid ethane. Grids were transferred to cryogenholders after vitrification and examined in a Philips electron microscope at 120 kV. The magnification was 17000.

NMR measurements, data processing and analysis

Samples containing an orienting medium were prepared as described in the corresponding paragraph. All experiments were carried out on a 600 MHz Varian Inova Spectrometer. The data were processed on a PC running Linux, using an NMR data processing package written in the language Python.

Acknowledgements

We thank Dr. M. Stuart for making the cryo-electron micrographs of the phospholipid mixtures.
<table>
<thead>
<tr>
<th>medium</th>
<th>orientation of shape</th>
<th>interactions macro-molecule</th>
<th>T range (°C)</th>
<th>pH range</th>
<th>ionic strength charge</th>
<th>special remarks</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC:DHPC</td>
<td>Perpendicular</td>
<td>swiss cheese</td>
<td>29 - 45</td>
<td>6-7</td>
<td>none</td>
<td>samples may phase separate</td>
<td>(Ottiger &amp; Bax, 1998)</td>
</tr>
<tr>
<td>DMPC:DHPC + PEG2000-PE</td>
<td>Perpendicular</td>
<td>swiss cheese</td>
<td>nd(40)</td>
<td>nd(7)</td>
<td>at least 0 - 600 mM NaCl</td>
<td>PEG-lipid headgroup bears a negative charge bilayers</td>
<td>(King et al, 2000)</td>
</tr>
<tr>
<td>DMPC:DHPC: CTAB</td>
<td>Perpendicular</td>
<td>swiss cheese, mainly steric</td>
<td>27 - ≥100</td>
<td>nd(6)</td>
<td>positive</td>
<td>stable up to 200 MPa; CTAB prevents phase separation</td>
<td>(Brunner et al, 2001;Losonczi &amp; Prestegard, 1998)</td>
</tr>
<tr>
<td>DMPC:DHPC: SDS</td>
<td>Perpendicular</td>
<td>swiss cheese</td>
<td>nd</td>
<td>nd</td>
<td>negative</td>
<td>SDS prevents phase separation</td>
<td>(Losonczi &amp; Prestegard, 1998)</td>
</tr>
<tr>
<td>DMPC:DHPC: DMPG or DMTAP</td>
<td>Perpendicular</td>
<td>swiss cheese</td>
<td>30-40</td>
<td>nd</td>
<td>[NaCl]&gt;50 mM; DMPG= negative; DMTAP= positive</td>
<td>charged amphiphiles prevent phase separation</td>
<td>(Crowell &amp; Macdonald, 1999)</td>
</tr>
<tr>
<td>14-O-PC:6-O-PC; 12-O-PC:6-O-PC</td>
<td>Perpendicular</td>
<td>swiss cheese</td>
<td>32 - 46</td>
<td>at least 1-12.5</td>
<td>none</td>
<td>temp. range varies with pH; ether linkages prevent hydrolysis</td>
<td>(Ottiger &amp; Bax, 1999)</td>
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<tr>
<td>DIO-DPC: CHAPSO</td>
<td>Perpendicular</td>
<td>swiss cheese</td>
<td>10 - ≥55</td>
<td>1 - 5</td>
<td>none</td>
<td>useful for molecules that are only stable at low pH</td>
<td>(Cavagnero et al, 1999)</td>
</tr>
</tbody>
</table>

Table 1: Overview of media used for orienting biomolecules. nd denotes that the exact range is not determined.

The value in brackets is the value used in the reference.
<table>
<thead>
<tr>
<th>medium</th>
<th>orientation of the normal</th>
<th>shape</th>
<th>interactions macromolecule</th>
<th>T range (°C)</th>
<th>pH range</th>
<th>ionic strength</th>
<th>charge</th>
<th>special remarks</th>
<th>references</th>
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</thead>
<tbody>
<tr>
<td>DLPC:CHAPSO</td>
<td>perpendicular</td>
<td>swiss cheese sheets</td>
<td>steric</td>
<td>7 - 50</td>
<td>nd (6.8)</td>
<td>nd (50 mM KCl)</td>
<td>none</td>
<td>useful for molecules that are only stable at low T</td>
<td>(Wang et al, 1998)</td>
</tr>
<tr>
<td>DLPC:DHPC</td>
<td>perpendicular</td>
<td>swiss cheese sheets</td>
<td>steric</td>
<td>nd (30)</td>
<td>nd</td>
<td>nd (100 mM NaCl)</td>
<td>none</td>
<td>by using DLPC instead of DMPC, the LC phase begins to form at lower T.</td>
<td>(Tan et al, 2002)</td>
</tr>
<tr>
<td>DMPC:DHPC + Ln³⁺</td>
<td>parallel</td>
<td>swiss cheese sheets</td>
<td>steric + electrostatic</td>
<td>35 - 90</td>
<td>nd</td>
<td>nd</td>
<td>positive</td>
<td>Ln³⁺ shifts and broaden NMR lines</td>
<td>(Prosser et al, 1998a)</td>
</tr>
<tr>
<td>DBBPC:DHPC</td>
<td>parallel</td>
<td>swiss cheese sheets</td>
<td>steric + electrostatic</td>
<td>10 - 54</td>
<td>nd</td>
<td>nd (100 mM NaCl)</td>
<td>none</td>
<td></td>
<td>(Tan et al, 2002)</td>
</tr>
<tr>
<td>CmEn/n-alkyl alchoh</td>
<td>perpendicular</td>
<td>lamellar sheets or concentric rings</td>
<td>mainly steric</td>
<td>-5 - 39</td>
<td>2 - 11</td>
<td>at least 0 - 500 mM NaCl</td>
<td>none</td>
<td>temp range depends on i) the type CmEn, ii) the salt conc. and iii) type and conc n-alkyl alcohol</td>
<td>(Ruckert &amp; Otting, 2000)</td>
</tr>
<tr>
<td>glucopone/ n-hexanol</td>
<td>perpendicular</td>
<td>lamellar sheets or concentric rings</td>
<td>steric</td>
<td>3 - 37</td>
<td>2 - 11</td>
<td>nd (0)</td>
<td>none</td>
<td>requires a long time to equilibrate</td>
<td>(Ruckert &amp; Otting, 2000)</td>
</tr>
<tr>
<td>Medium</td>
<td>orientation of the normal</td>
<td>shape</td>
<td>interactions macromolecule</td>
<td>T range (°C)</td>
<td>pH range</td>
<td>ionic strength</td>
<td>charge</td>
<td>special remarks</td>
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</tr>
<tr>
<td>CPCl/hexanol</td>
<td>perpendicular</td>
<td>Hellfrich phase or wormlike structures</td>
<td>mainly electrostatic</td>
<td>0 - 70</td>
<td>nd (30)</td>
<td>[NaCl] &gt;200 mM</td>
<td>positive at pH 7</td>
<td>requires a long time to equilibrate; samples remain aligned a few days after NMR exp.</td>
<td>(Prosser et al, 1998b)</td>
</tr>
<tr>
<td>CPBr/hexanol</td>
<td>perpendicular</td>
<td>Hellfrich phase or wormlike structures</td>
<td>mainly electrostatic</td>
<td>15 - 60</td>
<td>2 - 8</td>
<td>[NaBr] = 25 - 30 mM</td>
<td>positive at pH 7</td>
<td>easy sample recovery</td>
<td>(Barrientos et al, 2002)</td>
</tr>
<tr>
<td>Pfi phage</td>
<td>perpendicular</td>
<td>rods</td>
<td>steric + electrostatic</td>
<td>5 - ≥45</td>
<td>6.5 - 8</td>
<td>at least 0 - 500 mM NaCl; pI = 4.0</td>
<td>negative; surface charge result of OSO³⁻ groups</td>
<td>no critical lower threshold</td>
<td>(Hansen et al, 1998; Hansen et al, 2000)</td>
</tr>
<tr>
<td>purple membranes</td>
<td>parallel</td>
<td>disks</td>
<td>mainly electrostatic</td>
<td>&lt;69</td>
<td>2.5 - 10</td>
<td>[NaCl] &lt;70 mM</td>
<td>negative</td>
<td>easy sample recovery</td>
<td>(Koenig et al, 1999; Sass et al, 2000)</td>
</tr>
<tr>
<td>cellulose crystallites</td>
<td>parallel</td>
<td>chiral nematic phase</td>
<td>partially electrostatic</td>
<td>nd (37)</td>
<td>nd (5.2)</td>
<td>nd (0)</td>
<td>negative</td>
<td>surface charge result of OSO³⁻ groups</td>
<td>(Fleming et al, 2000)</td>
</tr>
<tr>
<td>LBT</td>
<td></td>
<td></td>
<td></td>
<td>nd (25)</td>
<td>nd (57)</td>
<td>nd (150 mM NaCl)</td>
<td>At the moment there are no details about the EDTA –based LBT.</td>
<td>(Wohnert et al, 2003a); <a href="http://medusa.nmr.mpibpc.mpg.de">http://medusa.nmr.mpibpc.mpg.de</a></td>
<td></td>
</tr>
</tbody>
</table>
Table 1 (continued): Overview of media used for orienting biomolecules.

<table>
<thead>
<tr>
<th>Medium</th>
<th>orientation of the normal</th>
<th>shape</th>
<th>interactions macromolecule</th>
<th>T range (°C)</th>
<th>pH range</th>
<th>ionic strength</th>
<th>charge</th>
<th>special remarks</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>stretched paa gels</td>
<td>perpendicular</td>
<td>prolate cavities</td>
<td>steric</td>
<td>at least 5  - 45</td>
<td>at least 2  - 8.5</td>
<td>at least 0 - 200 mM NaCl</td>
<td>none</td>
<td>easy sample recovery</td>
<td>(Sass et al., 2000; Tycko et al., 2000)</td>
</tr>
<tr>
<td>Compressed paa gels</td>
<td>parallel</td>
<td>oblate cavities</td>
<td>steric</td>
<td>at least 5 - 45</td>
<td>at least 2 - 8.5</td>
<td>at least 0 - 200 mM NaCl</td>
<td>none</td>
<td>easy sample recovery</td>
<td>(Sass et al., 2000; Tycko et al., 2000)</td>
</tr>
<tr>
<td>charged paa gels</td>
<td>perpendicular or parallel</td>
<td>prolate or oblate cavities, respectively</td>
<td>steric + electrostatic</td>
<td>at least 5 - 45</td>
<td>at least 2 - 8.5</td>
<td>at least 0 - 240 mM NaCl</td>
<td>positive or negative</td>
<td></td>
<td>(Meier et al., 2002; Ulmer et al., 2003)</td>
</tr>
<tr>
<td>paa stabilised gels (Pf1 or PM)</td>
<td>parallel</td>
<td>cavities + disks or rods</td>
<td>steric + electrostatic</td>
<td>see range paa and Pf1 or pm</td>
<td>see range paa and Pf1 or pm</td>
<td>see range paa and Pf1 or pm</td>
<td>Pfi or pm</td>
<td>orientation is permanent</td>
<td>(Sass et al., 2000; Trempe et al., 2002)</td>
</tr>
<tr>
<td>Pluronic F-127 stabilised gel (Pf1)</td>
<td>parallel</td>
<td>cavities + rods</td>
<td>steric + electrostatic</td>
<td>at least 20 - 30</td>
<td>nd (8)</td>
<td>nd (0)</td>
<td>see Pf1</td>
<td>suspension is liquid</td>
<td>(Riley et al., 2002)</td>
</tr>
<tr>
<td>V2O5</td>
<td>parallel</td>
<td>ribbons</td>
<td>mainly electrostatic</td>
<td>1 - 3</td>
<td>nd (0)</td>
<td>negative</td>
<td></td>
<td></td>
<td>(Desvaux et al., 2001)</td>
</tr>
<tr>
<td>H3Sb3P2O14</td>
<td>parallel</td>
<td>lamellar sheets</td>
<td>mainly electrostatic</td>
<td>4 - 50</td>
<td>2.5 - 9.5</td>
<td>[NaCl] &lt; 100 mM</td>
<td>negative</td>
<td>requires base + counterion</td>
<td>(Gabriel et al., 2001)</td>
</tr>
</tbody>
</table>
References


Chapter 2


zz with paramagnetic ions. *Journal of the American Chemical Society, 118*, 269-270.


Chapter 2


