

Chapter 4

Solid-state NMR study of microalgal membranes and cell walls

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Abstract

Microalgae are ubiquitous primary organisms that play key roles in the aquatic environment where they are the basis of the food web. These microorganisms also produce a variety of molecules with high added value, which find applications in human and animal nutrition, as therapeutic agents and as biofuels. Lipid membranes abound in microalgae where they protect the cell and are essential building blocks of organelles such as the chloroplast. To better understand the architecture and dynamics of microalgal cell membranes and their constituents, it is important that they are investigated in their natural environment. Solid-state nuclear magnetic resonance (SS-NMR) is emerging as a powerful tool to tackle these complex assemblies *in situ* on cell extracts and also in whole cells. Several nuclei can be studied to probe algal membranes and, moreover, microalgae can be fully ^{13}C - or ^{15}N -labeled in a cost-effective manner. In the case of full organism labelling, the challenge of SS-NMR is to develop and identify experiments to filter specific constituent signals. In this chapter, we show how SS-NMR can provide a wealth of information on the structure, dynamics and composition of microalgal membranes. We also summarize the main SS-NMR approaches so far applied on lipid extracts or whole cells under near to native conditions and suggest potential avenues to improve our understanding of these systems.

4.1 Overview

Microalgae are microscopic algal cells also known as phytoplankton. The cell wall and plasma membrane protect the organelles in the cytoplasm, such as the nucleus, the chloroplast, the reticulum, as well as the sugar and lipid reserves (figure 4.1). About 30 000 species of microalgae are found in fresh- and saltwater, where they are

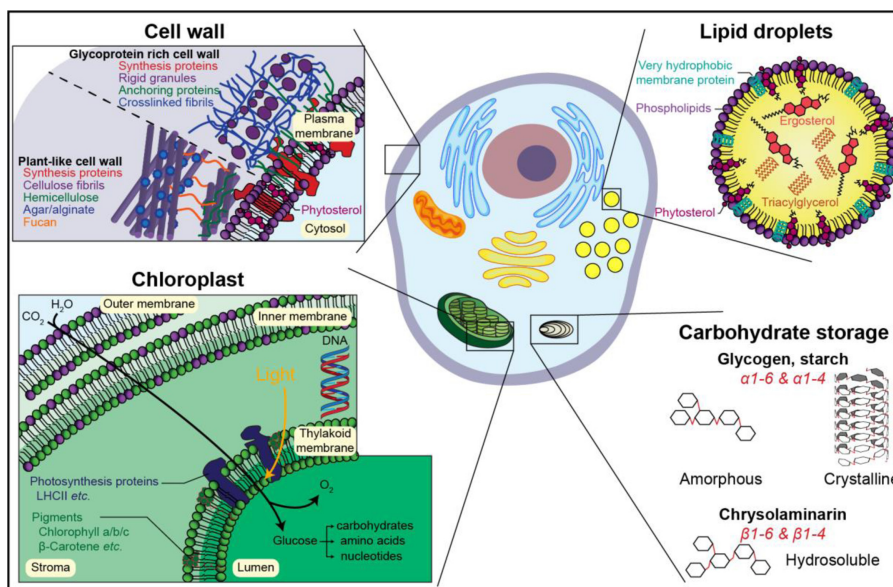


Figure 4.1. Microalgal cell with organelles and membrane structures. The cell wall is generally composed of glycoproteins, cellulosic material, carbonate or silica and the plasma membrane is rich in phospholipids. Chloroplast membranes have different galactolipid-to-phospholipid ratios, going from 19:81 in the chloroplast outer layer to 2:98 in thylakoid membranes (Harwood 1998). Lipid droplets incorporate TAGs and ergosterol, as a dense energy storage, surrounded by a single phospholipid monolayer. Energy can also be stored as complex carbohydrates, namely starch or chrysolaminarin.

at the basis of the aquatic food chain (Richmond and Hu 2013). As such, global warming or pollution effects on this resource can have an impact on all higher organisms.

Microalgae bear an important socio-economical potential. They are already used in human and animal nutrition, and have been exploited for their therapeutic activity related to antimicrobial (Falaise *et al* 2016), antiviral (de Jesus Raposo *et al* 2015), antioxidant, anti-inflammatory (Rasool *et al* 2007) or anti-cancer (Gutiérrez *et al* 2010) properties. Another interesting characteristic of microalgae is their ability to sequester and bioaccumulate heavy metals (Posadas *et al* 2017, Piccini *et al* 2019). They thus stand as an elegant avenue for contaminated water treatment. Microalgae are also used in the development of third-generation biofuels, which partially or completely come from solar instead of fossil energy (Abo *et al* 2019).

On a more fundamental level, microalgae are great tools for the study of photosynthesis. For example, carbon metabolism was deciphered in *Chlorella* by Melvin Calvin, who was awarded the Nobel Prize in 1961. The dynamics of photosynthesis has been investigated in detail in *Chlamydomonas* (Eberhard *et al* 2008). New transgene strategies are available, mainly studies of *Chlamydomonas reinhardtii* (Doron *et al* 2016, Baier *et al* 2018), which has been called the ‘photo-synthetic *Escherichia coli*’ because of its optional heterotrophic metabolism, allowing the use of non-photosynthetic mutants.

The microalgal cell wall, membranes and lipids are key players for both biochemical processes and biotechnological applications. Their selective permeability and mechanical or osmotic shock resistance make the cell interfaces and membranes a source of inspiration for biomaterial development. Furthermore, lipids in general are valuable nutritional ingredients with long-chain and ω -3 polyunsaturated fatty acids (FAs), but also as precursors for biodiesel production from triacylglycerol (TAG). Understanding the composition, dynamics and architectures of these assemblies is essential to bioengineer new microalgal strains with efficient photosynthesis and easy lipid extraction from the biomass, or to design novel materials based on natural products with interesting biological/physical properties. Finally, microalgal membrane structure and composition (see figure 4.1) can vary depending on temperature, pH or interaction with exogenous molecules, making them excellent global warming reporters.

Solid-state nuclear magnetic resonance (SS-NMR) is an invaluable tool providing nanoscopic information on the structure, dynamics and interactions of large biological molecules with virtually no limit in molecular size. This is why SS-NMR has proven its capabilities to investigate whole cell constituents, as well as their organization and membrane interactions. However, the cell complexity leads to crowded NMR spectra in which many signals potentially overlap. Therefore, SS-NMR approaches are being developed to tackle such challenging systems (Warnet *et al* 2015, Werby and Cegelski 2019, Narasimhan *et al* 2020). Considering the environmental and economical importance of microalgae, the objective of this chapter is to present how SS-NMR can provide information on the molecular composition and organization of microalgal membranes and the cell wall. Before presenting different NMR methodologies to address these cell compartments, the composition of microalgal membranes will first be summarized.

4.2 Microalgal cell membrane composition and architecture

Microalgal membranes are composed of lipids, proteins, carbohydrates, phytosterols as well as pigments. Although lipids ensure membrane integrity and efficient cell impermeability, the detailed lipidome of microalgae has been described for only a handful of species such as *C. reinhardtii* and *Nannochloropsis oceanica* (Zhao *et al* 2013, Nguyen *et al* 2013). Membranes are also rich in proteins that maintain the cell structure and metabolism, and in carbohydrates bound to both lipids and proteins, allowing key processes such as cell recognition or protein activity. Pigments are found in chloroplast membranes (figure 4.1) where they are involved in light energy capture and dissipation. Indeed, a lipid membrane also protects organelles in the cytoplasm, such as the reticulum and the nucleus. Moreover, lipid droplets are also present inside the cells.

4.2.1 Microalgal cell wall and plasma membrane

The cell wall primarily acts as a shell and thus makes biomolecules such as TAGs harder to harvest and exploit. It also protects the cell from mechanical constraints and external stress, and is involved in cell-cell and cell-substrate adhesion,

protection from bacteria as well as sexual reproduction (Domozych *et al* 2012). Although the plasma membrane is not the largest membrane in the cell, it must be studied since it faces a wide range of stresses such as temperature changes and osmotic shocks in its environment. Moreover, multiple specific transporters are found within this membrane, which is the last barrier crossed by molecules before accessing the cytosol. It is also where cell signalling is initiated. One of the first studies of the lipid composition of microalgal plasma membranes focused on the green algae *Hydrodictyon africanum*. Phospholipids, such as phosphatidylcholine (PC), phosphatidylserine (PS) and cardiolipin (CL), were mainly identified (Bailey and Northcote 1976). The microalgal cell membrane is mostly composed of polar lipids (Harwood 1998) and its composition can adapt to external stress or cell state (Mimouni *et al* 2018).

The relationship between the plasma membrane and the microalgal cell wall is so intimate that considering the membrane without the cell wall would make no sense in terms of dynamics and shape. Indeed, the cell wall synthesis and its remodelling proteins are associated to the membrane (Popper *et al* 2014) and some fibrillar assemblies anchored in the cell wall traverse the plasma membrane (Domozych 2019), making membrane and cell wall boundaries hard to define precisely (figure 4.1). For microalgae such as Chlorophyceae, the cell wall is composed of very organized carbohydrates and glycoproteins (Rashidi and Trindade 2018), so that glycan patterns and structures are important to determine. While electron microscopy (EM) has provided micrometer resolution images important for taxonomic determination, SS-NMR can potentially provide additional structural and molecular-level dynamic information of the cell membrane on different timescales.

4.2.2 Photosynthetic organelles (thylakoids and chloroplast)

There are several organelles in the algal cell and a membrane borders them all. The chloroplast has, by far, the largest membrane surface, with three layers of densely packed lipid membranes (figure 4.1). This explains why the lipid profile of other organelles is difficult to determine without getting any chloroplast membrane or pigment contamination during purification. The lipid and FA chain profiles of microalgae are usually given for the whole organism (figure 4.2). The membrane surface of each organelle has not been measured or estimated yet, even if a 3D reconstitution made by *in situ* cryo-electron tomography of *C. reinhardtii* has been reported (Engel *et al* 2015).

The lipid composition of chloroplasts membranes is very different from that of plasma membranes as they mostly contain galactolipids and few phospholipids. It varies according to the species but monogalactosyldiacylglycerol (MGDG, 40 to 55%), digalactosyldiacylglycerol (DGDG, 15%–35%), sulfoquinovosyldiacylglycerol (SQDG, 10%–20%) and phosphatidylglycerol (PG, 10%–20%) are the main components (Harwood 1998). In *Chlamydomonas*, the chloroplast envelopes and thylakoid membranes have slightly different lipid compositions (Mendiola-Morgenthaler *et al* 1985). On the other hand, MGDG/DGDG and SQDG/PG

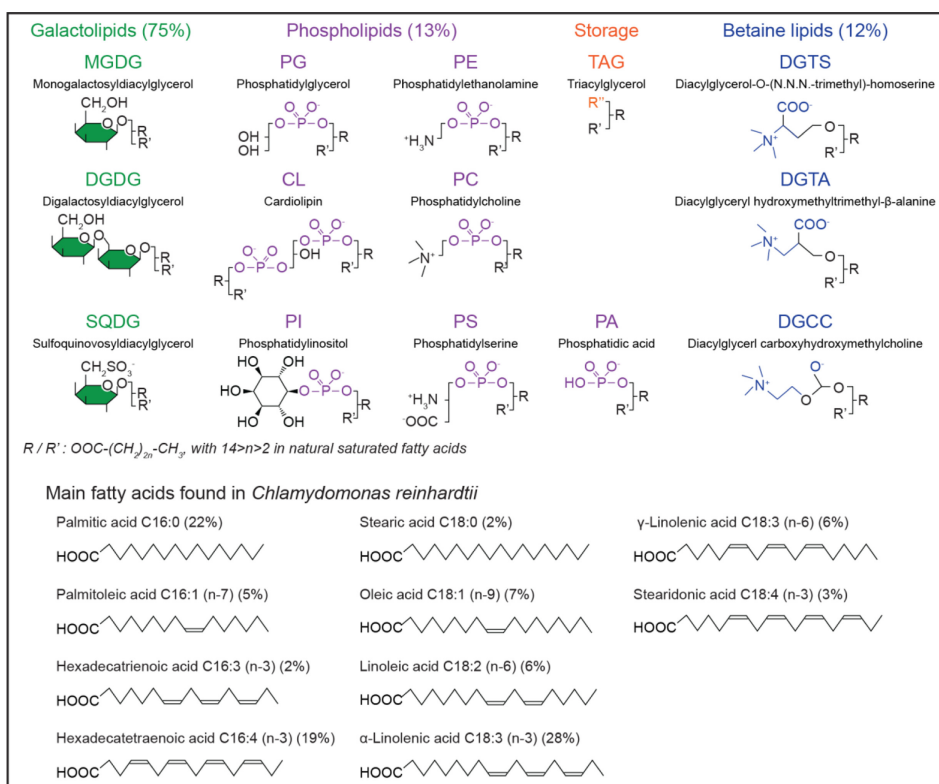


Figure 4.2. Main lipids and fatty acids found in microalgae. Galactolipids and betaine lipids are the most abundant in microalgae, and mainly located in photosynthetic membranes. Phospholipids are found in other membranes. Triacylglycerols (TAGs) are stored in lipid droplets. The lipid profile and fatty acid chain composition are species- and medium-dependent. As a reference, values for *C. reinhardtii* are indicated (Vieler *et al* 2007, Nguyen *et al* 2013).

ratios seem to be regulated in chloroplast membranes of various species and could be critical for proper thylakoid development (Boudière *et al* 2014). Betaine lipids have been detected in a few brown algae, such as diacylglycerol-O-(N,N,N-trimethyl) homoserine (DGTS) and diacylglycerol-O-(hydroxymethyl)-(N,N,N-trimethyl) β -alanine (DGTA) (Murakami *et al* 2018). Finally, recent work mentions glycolipids as microalgal membrane stabilizers because, during water or cold stress, the chloroplast membrane can be enriched in oligogalactolipids, produced by MGDG conversion (Hölzl and Dörmann 2019).

4.2.3 lipid droplets and free fatty acids

The lipid droplet (LD) is an important organelle for biofuel production due to the TAGs that they contain. Little is known about microalgae LDs but interest is growing due to their high potential as an alternative to fossil fuels. TAGs produce twice as much energy than starch and protein per unit weight. Moreover, microalgae

are of great interest for nutrition purposes because of the essential long-chain and unsaturated FAs that are contained in the LDs (Spolaore *et al* 2006).

The number, size and composition of LDs vary greatly among microalgal cell types but also according to the life cycle and metabolic stress. For example, nitrogen, iron, sulfur and phosphorus starvation are commonly used strategies to increase lipid storage in microalgae such as *C. reinhardtii* (Hu *et al* 2008, Wang *et al* 2009, Goold *et al* 2014). The size of LDs can range from nm to μm and they are very dynamic in terms of molecular motion, anabolism and catabolism. LDs are easily observed in the cytosol using lipophilic dyes such as Nile red or Bodipy, thus facilitating their quantification by microscopy or flow cytometry, as well as transmission electron microscopy which has been used to further describe their structure (Goodson *et al* 2011). Lipid droplets share the same overall structure with a hydrophobic oil core of storage lipids that mainly comprise TAGs (80%–90% by weight) and sterol esters, surrounded by a phospholipid monolayer that also contains proteins (only 5% by weight) (figure 4.1). Biochemical and microscopy studies have shown that the endoplasmic reticulum and plastid membranes are involved in LD production (Fan *et al* 2011, Goodson *et al* 2011). LDs are also associated with lipid homeostasis and several cellular signalling processes (Farese and Walther 2009).

The FA composition of TAGs is similar to that of whole cells (Goold *et al* 2014). However, the profile of the LD enveloping monolayer is very specific. For example, in *C. reinhardtii* (Wang *et al* 2009) and *Haematococcus pluvialis* (Peled *et al* 2011), DGTS, sulfoquinovosyldiacylglycerol (SQDG) and traces of DGDG are its major component. The lipids around the LD core are thought to be intermediates for acyl chain production in TAGs. Specific proteins are associated with LDs (Yoneda *et al* 2016, Wang *et al* 2017, Wang *et al* 2019). In *C. reinhardtii*, the predominant protein is the very hydrophobic major lipid droplet protein (MLDP) (Goold *et al* 2014), but other LD-associated proteins have been identified (Vieler *et al* 2012), and complete proteomics describe more than 200 proteins linked to LDs (Nguyen *et al* 2011).

4.3 Technical considerations

All the microalgal cell compartments presented in the previous section have a large diversity in molecular composition and structure. However, sophisticated techniques and appropriate stable isotope labelling allow the determination of molecular structures, dynamics and interactions in complex systems such as microalgae by SS-NMR.

4.3.1 Observable nuclei for microalgal membranes studies

Since protons (^1H) are the most sensitive nuclei for biological applications in NMR, ^1H -based techniques have been used to study microalgae, as will be illustrated below. The large abundance of protons in biological samples is an advantage in terms of sensitivity, but a drawback in terms of resolution. Therefore, other nuclei with higher specificity are frequently exploited. Phosphorous-31 (^{31}P), for example, has 100% natural abundance and high gyromagnetic ratio. Phosphorus is mainly

found in ATP, DNA, phosphorylated proteins but also in phospholipids and, therefore, ^{31}P NMR is an excellent probe to determine the membrane composition and dynamics (Overall *et al* 2019). Carbon is present in all organic molecules of the cell but only the ^{13}C isotope, which has a low (1%) natural abundance, generates an NMR signal. Since its gyromagnetic ratio is one quarter that of ^1H , ^{13}C has a lower sensitivity and isotopic enrichment is required for experiments to be time efficient. Finally, ^{15}N NMR can also be used to study microalgae, but is even less sensitive than ^{13}C and with very low natural abundance (0.4%), so ^{15}N NMR can only be performed with isotopic labelling.

Uniform ^{13}C - and/or ^{15}N -labelling of microalgae is easily carried out with affordable carbon and nitrogen sources, i.e., sodium bicarbonate ($\text{NaH}^{13}\text{CO}_3$) and ammonium chloride ($^{15}\text{NH}_4\text{Cl}$), respectively. The cost of growing uniformly labelled *C. reinhardtii*, for example, can be estimated to less than 100\$/L of medium with an optimal cell concentration of 3 to 4×10^6 cells/mL. Depending on metabolism or the targeted molecule, the medium might also require more expensive ^{13}C -labelled glucose, sodium acetate (Venters *et al* 1991) or CO_2 (Akhter *et al* 2016).

Targeted labelling of specific molecules of interest, such as lipids, amino acids, carbohydrates or pigments, within the whole organism is an interesting alternative approach. This strategy can be applied to ^{13}C or ^{15}N but also to other reporter nuclei, such as ^2H (Pius *et al* 2012, Tardy-Laporte *et al* 2013, Warnet *et al* 2016, Molugu *et al* 2017, Salnikov *et al* 2019) (see chapter 4) or ^{19}F (Matsumori *et al* 2008, Shi *et al* 2012, Afonin *et al* 2014, Kozorog *et al* 2018), often chosen to replace protons in key locations. These specific labelling approaches have rarely been applied to microalgae as they require a good knowledge of algal metabolism, but they hold great promise in particular for *in vivo* SS-NMR studies, as performed with intact bacteria (Booth *et al* 2017).

4.3.2 Solution and solid-state NMR

The NMR line width of a given nucleus depends on its interactions with neighbouring nuclei or electrons, including dipolar and quadrupolar interactions, chemical shift anisotropy but also anisotropic magnetic susceptibility effects. Most of these interactions are orientation-dependent but averaged out under fast and isotropic tumbling, such as for small molecules in solution, resulting in very narrow NMR lines.

In solution NMR, molecules can be discriminated according to their diffusion correlation time. Such spectral filtering hence simplifies the spectra and has been applied to study whole microalgal cells (Courtier-Murias *et al* 2012, Akhter *et al* 2016). Among the available ‘diffusion filters’, *diffusion editing* (DE) removes the signal of mobile molecules, while *inverse diffusion editing* (IDE) only shows the spectrum of fast diffusing molecules. Single pulse experiments will obviously show both types of signals.

Macromolecules and complex assemblies such as cell extracts, or even whole cells such as microalgae, are considered as ‘solid’ on the NMR time scale. The orientation dependence of the interactions presented above leads to broad lines that often

overlap with neighbouring signals, resulting in the loss of spectral resolution. Such systems are thus studied using SS-NMR techniques. High resolution can be restored by using strategies that replace fast and isotropic tumbling by coherent averaging. One such strategy is magic-angle spinning (MAS), where the sample is spun at high frequency at an angle of 54.7° with respect to the magnetic field direction. Depending on the available spinning frequency, interactions can be completely averaged out, resulting in narrower lines. The required spinning frequency depends on the observed nuclei and the interactions that they experience. One may wonder if high spinning frequencies would be deleterious to soft eukaryotic cells. Microalgae are very resistant organisms, owing to their protective and rigid cell wall. It has been shown that *C. reinhardtii* can spend 24 h in an SS-NMR rotor spinning at 10 kHz, at room temperature and without any nutrients, with around 80% survival and cell morphology preserved (Poulhazan *et al* 2018).

In the case of protons in rigid solids, homonuclear dipolar interactions are very strong and often cannot be efficiently averaged out using standard MAS. Therefore, most ^1H MAS SS-NMR studies are restricted to very dynamic small molecules where the remaining dipolar couplings and anisotropic magnetic susceptibility can be cancelled by MAS at moderate frequencies (typically below 10 kHz). This is the field of high-resolution magic-angle spinning NMR (HRMAS). In ^1H HRMAS, as in solution NMR, the lipid droplet signals dominate those of membrane lipids, whereas higher spinning frequencies would be required to observe intense membrane phospholipid, carbohydrate or protein signals. HRMAS allows for direct measurements of non-liquid soft tissues and cells, generally by ^1H NMR, and is typically used to characterize metabolites, even in intact cells, as is summarized in the in-cell section.

High-resolution SS-NMR developments over the last 30 years have provided robust methodologies to characterize the structure and dynamics of biological membrane components (lipids and proteins) by observing ^{13}C signals under MAS, sometimes in combination with ^{15}N since these nuclei are found in all peptide bonds. Since the internuclear interactions involved are smaller than for ^1H , standard MAS is often enough to generate high-resolution one- (1D) and multidimensional spectra. In some cases, line narrowing by MAS results in membrane components spectra that are similar to those obtained by solution NMR.

While solution NMR and HRMAS are restricted to molecules with ‘fast’ dynamic properties, SS-NMR can reveal and exploit the dynamic heterogeneity of biological membranes. To do so, adequate ‘dynamic filters’ that select only rigid or dynamic components within the sample are utilized. This results not only in spectral simplification but also provides information regarding the molecular environment. The most common filters take advantage of proton-to-carbon polarization transfer schemes. For example, INEPT (nuclei enhancement by polarization transfer) will only be efficient for very dynamic molecules (with tumbling times from 10^{-15} to 10^{-9} s) and only detect carbons directly bound to protons. On the other hand, cross polarization (CP) will only show carbons from more rigid molecules (with correlation times from 10^{-9} to 1 s). Single pulse ^{13}C experiments will show both types of signals. Other types of filters, that will not be

detailed here, have also been developed (Reckel *et al* 2012, Renault *et al* 2012, Warnet *et al* 2015, Matlahov and van der Wel 2018).

Since cells are not pure solids or pure liquids, the research group of Simpson has developed tools to study such heterogeneous samples with an approach called *comprehensive multiphase* (CMP) NMR (Simpson *et al* 2013, Liaghati Mobarhan *et al* 2019). Indeed, the dynamical heterogeneity of intact cells requires the use of both solution and SS-NMR approaches to obtain a complete picture of their composition and function. CMP-NMR probes thus combine lock, gradients and improved shimming capabilities, typical of solution NMR probes, with access to moderate magic-angle spinning frequencies (*vide infra*) of a few kHz and high-power pulses typical of SS-NMR probes. With this type of equipment, monitoring different types of molecules is possible: from dissolved metabolites up to very rigid storage molecules or proteins.

4.4 NMR of lipid extracts and model membranes

Considering the complexity of microalgal cells, simple and straightforward approaches are used to garner information on membrane lipids by NMR. First, lipids can be extracted, dissolved in organic solvents, and studied by simple ^1H , ^{13}C , or ^{31}P solution NMR. This approach has been applied, for example, on whole cell extracts of *Pavlova spp.* (Kobayashi *et al* 2007) or *Neochloris oleoabundans* (Beal *et al* 2010) for lipid assignment and quantification in combination with other techniques (Challagulla *et al* 2017). The use of lipid extracts obviously neglects important information such as the lipid environment, collective properties and membrane dynamics.

Another strategy to study microalgal membrane properties is to employ model systems with lipid composition similar to the mimicked membranes. Although part of the living organism complexity is sacrificed, model membranes provide valuable information on specific biophysical properties and interactions with the lipids. Mixtures of galactolipids such as MGDG and DGDG have been studied by ^2H and ^{31}P static SS-NMR as early as 1985 to investigate phase transitions in microalgae and chloroplast membranes (Brentel *et al* 1985, Lindblom *et al* 1986, Borovyagin *et al* 1988). Galactolipid structures and dynamics were later probed by ^{13}C SS-NMR using MAS (Adebodun *et al* 1992, Gross *et al* 1995, Castro *et al* 2007) or in fast-tumbling bicelles by solution NMR (Howard and Prestegard 1995, Ye *et al* 2013). Figure 4.3 shows a ^1H – ^{13}C proton-detected local field (PDLF) study that allowed measurement of the order parameters at various carbon positions in model unlabelled DMPC/MGDG membranes. This revealed that MGDG reduced the order parameters along DMPC molecules (Castro *et al* 2007). Simplified thylakoid model membranes made of palmitoyl-oleoyl phosphocholine (POPC) and MGDG were used to determine the interactions of Hcf106—a peptide found in the thylakoid of microalgae—with its host membrane lipids (Zhang *et al* 2013, Zhang *et al* 2014). Finally, the light harvesting complex II (LHCII) of *C. reinhardtii* was also studied by ^{13}C , ^{15}N -NMR reconstituted in a thylakoid-like membrane environment made up of MGDG, DGDG, SQDG and PG (Azadi-Chegeni *et al* 2018b).

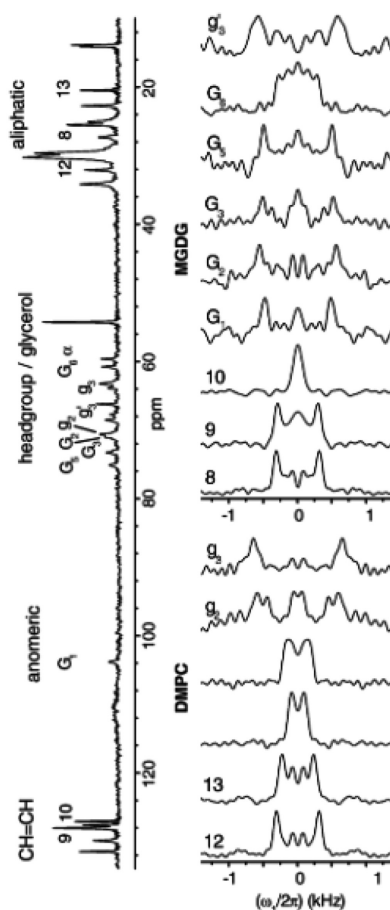


Figure 4.3. ^1H - ^{13}C MAS (5 kHz) NMR PDLF spectra of a model membrane made of unlabelled DMPC and MGDG, recorded at 42 °C. The 1D ^{13}C NMR spectrum is displayed on the left. Reprinted from Castro *et al* (2007) with permission from Elsevier.

4.5 *In vivo*, in-cell and *in situ* NMR

In-cell SS-NMR tackles complex samples that are very hard to characterize using other atomic-resolution techniques such as EM, x-ray crystallography or solution NMR. While *in vivo* NMR addresses the challenge of studying living cells, *in situ* NMR examines cell extracts—the cell is no longer intact but molecules are still in their native local environment. *In vivo*, in-cell and *in situ* SS-NMR have only recently been applied to microalgae but a wide range of applications are possible, as will be presented in the next sections.

4.5.1 ^1H -NMR based approaches

As previously explained, HRMAS provides insights into cellular metabolism by producing spectra with resolution sometimes comparable to that of solution NMR;

and also allows spectral editing according to the dynamics of the constituents. HRMAS is applied to a variety of biological samples including human or mice cell lines, or tissues for clinical studies, allowing diagnosis and follow up of organism responses to treatments (Kaebisch *et al* 2017). It is a common tool in metabolomics, especially for detecting metabolites associated with lipids such as inositol, phosphoryl choline, FAs and cholesterol. ^1H HRMAS is also used in lipid profiling and characterization of the saturated/unsaturated FA ratio (Griffin *et al* 2003, Kabli *et al* 2009, Berry *et al* 2016). Terpenes found in chloroplast membranes are interesting molecules also detected by HRMAS (Gaysinski *et al* 2015). Finally, because lipids are an important part of cell metabolism, several studies have focused on alterations of lipid metabolism using HRMAS to detect lipid droplet perturbation, for example in cancer cell lines (Pan *et al* 2012).

Considering the capabilities of HRMAS, this approach has been used to study microalgae, including their membranes and the *in vivo* intracellular C/N ratio (Bondu *et al* 2008, Hashim *et al* 2013). Native membranes are difficult to study because they are rigid structures on the NMR time scale, thus leading to broad and overlapping lines, with notable exceptions (Simon *et al* 2015). Merkley and Syvitski (Merkley and Syvitski 2011) successfully assigned lipids and carbohydrates in whole *Nannochloropsis granulata* and monitored the unsaturated/saturated FA ratio as a function of cell harvest time using multidimensional ^1H - ^{13}C HRMAS. Also, Chauton *et al* published several studies on different microalgae species, such as *C. mülleri* (Webb *et al* 2006), *Dunaliella* sp. (Chauton and Størseth 2008), *Chaetoceros muelleri* (Chauton *et al* 2004) and *Thalassiosira pseudonana* (Chauton *et al* 2003). In these works, detailed assignments of lipids were reported, allowing quantification in response to growth conditions. HRMAS was also used to classify microalgae using statistical data treatments such as principal component analysis (PCA). NMR studies performed on other cells and organisms will certainly inspire future investigations of microalgal lipids. For example, HRMAS has been used to characterize erythrocyte membranes and perform permeability studies in *Daphnia magna* (Bruno *et al* 2006, Kovacevic *et al* 2018). Recent work on living earthworms (*Eisenia fetida*) and freshwater shrimps (*Hyalella azteca*) have improved the lipid signal treatment based on relaxation and diffusion spectral filtering (Mobarhan *et al* 2016, Hassan *et al* 2019).

An alternative to HRMAS studies for ^1H detection is the emerging use of ultrahigh frequency MAS. Although membrane lipids and proteins can be studied at intermediate frequencies (15 kHz (Gopinath *et al* 2017)), ultra-fast MAS (>50 kHz) allows detection of protons in rigid molecules with increased sensitivity and a resolution comparable to that of solution NMR. Combined with ^{13}C and ^{15}N labelling of samples, sequential assignment of membrane proteins or other complex molecules is possible, even in their native environment, e.g. proteins involved in photosynthesis (Beal *et al* 2010, Medeiros-Silva *et al* 2016). New information on biomembrane structure is expected to emerge from this approach, in particular for the study of membrane proteins in microalgae.

4.5.2 1D ^{13}C solid-state NMR methods

As stated above, SS-NMR can be exploited to obtain valuable information on cell samples. Depending on the spectral editing used, both rigid and dynamic molecules can be identified and their dynamics *in situ* revealed—information that is lost using solution NMR of cell extracts, for example. Whole uniformly ^{13}C -labelled *C. reinhardtii* were studied by our group in 2015 by 1D ^{13}C SS-NMR with MAS, along with *Pavlova lutheri* and *Nannochloropsis oculata* (Arnold *et al* 2015). This work allowed the assignment of different lipids, carbohydrates including glycans, as well as rigid cell-wall proteins using different dynamic filters. As shown in figure 4.4, the more mobile molecules of *C. reinhardtii* are revealed by ^{13}C SS-NMR using the INEPT filter, the most intense peaks being ascribed to methyl groups from the lipid acyl chains around 30 ppm. This spectrum is comparable to a ^{13}C solution NMR spectrum using the IDE filter, with a slightly lower resolution.

On the exact same sample, a CP filter revealed the more rigid molecules such as starch, with C1 carbon at 101 ppm. Starch is invisible in solution NMR, even with a DE filter. These experiments highlight that many lipids are very mobile, while proteins in the cell wall are much more rigid assemblies. In 2018, Azadi-Chegeni *et al* published similar work on *C. reinhardtii* whole cells (Azadi-Chegeni *et al* 2018a, Azadi-Chegeni *et al* 2018b). For example, they assigned several component resonances in whole cell samples and quantified the dynamics in terms of correlation times and order parameters of these molecules at different temperatures (Azadi-Chegeni *et al* 2018a). They also investigated LHCII in native membranes and showed that they favored protein conformational stability. These works represent a step toward the understanding of chloroplast complexity in terms of molecular composition and dynamics.

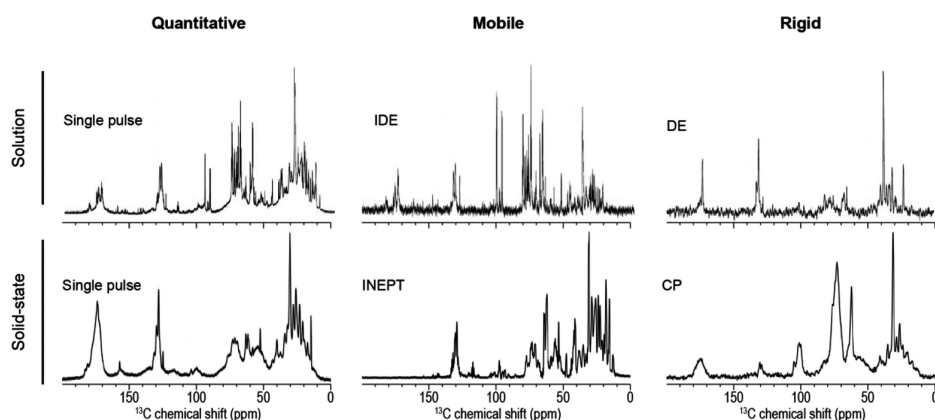


Figure 4.4. Comparison of ^{13}C labelled *C. reinhardtii* whole cell spectra by solution and solid-state NMR using either single pulse or filtered experiments. Solid-state experiments were performed at 600 MHz at 25 °C with a 10 kHz MAS frequency (adapted from Arnold *et al* (2018)). Solution NMR experiments were performed at 500 MHz at room temperature (adapted from Akhter *et al* (2016)). Reproduced with permission from Springer.

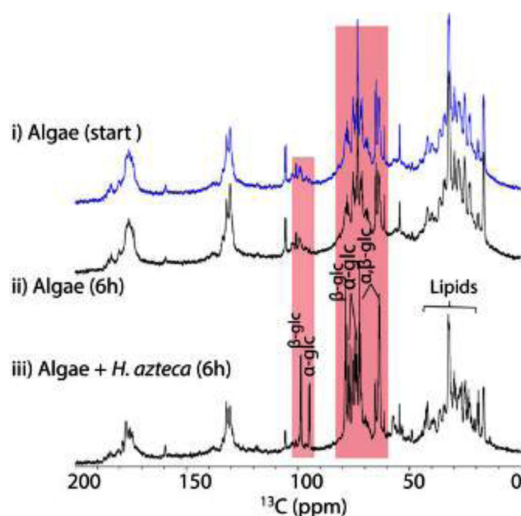


Figure 4.5. ^{13}C CMP NMR (15 °C and 1.1 kHz spinning frequency) spectra of uniformly ^{13}C -labelled *Chlorella vulgaris* microalgae with and without *H. azteca* daphnia. The pink region highlights the glucose peaks that increase when *H. azteca* is in contact with the algae. Reprinted from Liaghati Mobarhan *et al* (2019) with permission, copyright (2019) John Wiley & Sons.

Another interesting study reports the consumption of *Chlorella vulgaris* lipids by the daphnia *Hyalella azteca* using comprehensive multiphase NMR and ^{13}C -labelled microalgae. As shown in figure 4.5, good resolution was obtained and lipid consumption could be monitored with this new probe. Multidimensional solid-state experiments were also recorded with ^1H - ^{13}C correlations from ^{13}C -labelled algae and ^2H - ^{13}C correlations from ^2H - ^{13}C uniformly labelled algae.

4.5.3 2D- ^{13}C and ^{15}N -NMR experiments

With isotopically-labelled samples sensitivity is greatly increased, enabling the use of multidimensional SS-NMR which also significantly enhances spectral resolution. 2D SS-NMR NMR has already proven successful in studying biological membranes *in situ* or in whole cells, mainly bacteria (Andronesi *et al* 2005, Schanda *et al* 2014, Romaniuk and Cegelski 2015, Booth *et al* 2017, Bouhlef *et al* 2019) but also bovine retinas (Verhoeven *et al* 2001). More closely related to algal membranes, the work on bacterial chlorosomal antenna by Van Rossum *et al* is the first to mention and assign galactolipid signals in a native thylakoid membrane protein study (van Rossum *et al* 2001).

As proteins are part of the membrane architecture, it is important to present some of the SS-NMR studies carried out on these proteins in microalgal membranes. Protein conformations, dynamics and interactions with different chloroplast partners in photosynthesis have been described in several model organisms in a membrane environment using multidimensional SS-NMR, for example by the De Groot group, for green and purple bacteria light harvesting complexes (Huang and McDermott 2008, Alia *et al* 2009) but also bacteriorhodopsin (Varga *et al* 2008).

They have also shown the interaction between photosynthesis-associated proteins and their chromophore, using a single amino acid labelling approach (Alia *et al* 2001). To better understand the photosynthesis machinery in an environment close to native, the LHCII protein complex was purified from *C. reinhardtii* with some residual lipids and examined by multidimensional SS-NMR (Pandit *et al* 2011, Pandit *et al* 2013, Sunku *et al* 2013). LHCII switching its conformation from light harvesting to photoprotective mode through non-photochemical quenching was studied under MAS (<15 kHz) NMR and at temperatures below -30°C .

The only multidimensional SS-NMR study of a light harvesting system in native microalga membranes, without any purification or reconstitution steps, was performed on isolated *C. reinhardtii* thylakoid membranes (Pandit *et al* 2013). The resolution was sufficient to report LHCII conformational dynamics—a phenomenon impossible to observe using detergents or protein crystals. The results are thus specific to the dynamics in membranes of a photosynthetic organism, which prevents photo-damage. Figure 4.6 compares ^{13}C – ^{13}C spectra of LHCII in its native thylakoid environment and reconstituted in detergent. This can be compared also to ^{13}C – ^{13}C spectra of the same protein extracted from its membrane or reconstituted in a model thylakoid-like membrane, as done by Azadi-Chegeni *et al* (2018b).

By using *in vivo* 2D solid-state NMR and dynamic spectral edition on ^{13}C -labelled microalgae, we improved our previous 1D NMR results for *C. reinhardtii*, showing that

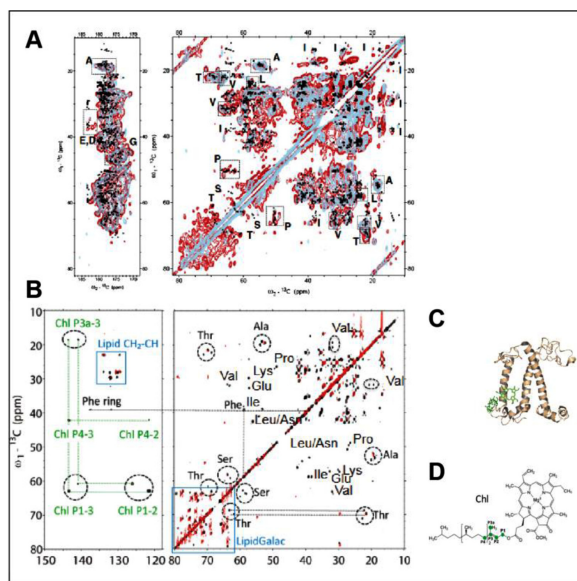


Figure 4.6. ^{13}C – ^{13}C solid-state NMR spectra of LHCII from *C. reinhardtii* in: (A) its native thylakoid environment (red contours), reconstituted in β -n-dodecyl-D-maltoside detergent buffer (light blue contours); and (B) pure extracted protein (black contours) and in a reconstituted thylakoid membrane (red contours). (C) The LHCII crystal structure, and (D) chlorophyll molecular structure are also given. A is adapted from Pandit *et al* (2013) with permission from Elsevier, and B–D are adapted from Azadi-Chegeni *et al* (2018b), with permission.

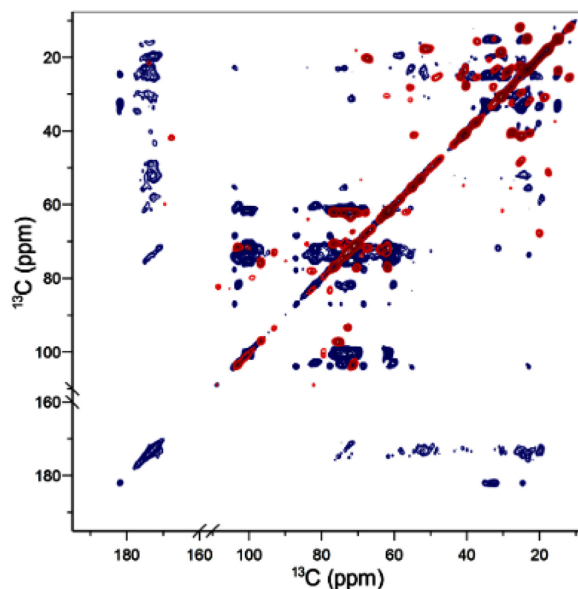


Figure 4.7. ^{13}C – ^{13}C *in vivo* solid-state NMR spectra of uniformly ^{13}C -labelled *C. reinhardtii* recorded at a MAS frequency of 26.7 kHz using either INEPT- (in red) or CP-based (in blue) filters. From Arnold *et al* (2018) with permission from Springer.

precise assignments of carbohydrates, lipids and proteins are possible (Arnold *et al* 2018). Figure 4.7 compares rigid and mobile molecules detected by ^{13}C – ^{13}C SS-NMR using either INEPT- or CP-based filters. A large range of microalgal membrane lipid classes could thus be identified *in situ*, with those from thylakoid membranes dominating due to their abundance. In addition to lipids and rigid proteins, assignment and characterization of carbohydrates *in situ* is a necessary step towards a better understanding of carbohydrates architecture in the cell walls of microalgae, and their ability to face osmotic shocks, for example.

4.6 Conclusion and future prospects

In this chapter, we have reviewed the NMR techniques that can provide molecular-level information on the structure, as well as dynamics, of microalgal membranes constituents. So far, little is known about these membranes reflecting their overall complexity but they are a key component to understanding the photosynthetic machinery, and to bioengineer new strains for biofuel or food supplements.

We have shown the versatility of NMR, which can detect nuclei, such as ^1H , ^{31}P , ^{13}C , ^{15}N , ^2H and ^{19}F , in either single or multidimensional experiments. It is applicable to small or large, dynamic or rigid molecules, to metabolites, model membranes, cell extracts, whole cells and even living cells. For NMR, isotopic labelling is generally required. While uniform labelling is routinely performed, as demonstrated here with examples of fully ^{13}C -labelled microalgae, new approaches can certainly be developed to specifically label molecules of interest within whole

microalgae, providing that the biosynthetic pathways are known. This strategy has notably been applied to bacteria and to ^2H *in vivo* SS-NMR study of their membranes (Booth *et al* 2017).

Depending on the sample and cellular constituent studied, solution or SS-NMR approaches are available. In the solid state, they include static or MAS samples, spinning at moderate or high frequencies. New techniques continue to emerge, including the use of dynamic filters and comprehensive multiphase NMR, which are useful to discriminate membrane lipids from lipid droplets, for example.

Beside broad molecular identification, NMR focuses on high-resolution structure determination of biomolecules. Microalgal membrane proteins and photosynthetic systems are important targets since they are involved in metabolism and transport. Their structures will need to be determined to understand these mechanisms (Ladizhansky 2017). Revealing the structure of microalgae cell walls (including their connexion to the membrane) is also an area where SS-NMR can contribute, either within whole cells or extracts, as has been done with higher plants to obtain atomic resolution of cellulose nanostructures (Dick-Pérez *et al* 2011, Wang and Hong 2015). Such information is essential for a fundamental understanding of microalgal species and also for commercial exploitation of this aquatic resource.

Finally, dynamic nuclear polarization (DNP) coupled with SS-NMR has been shown to be very powerful for studies of biological assemblies, such as bacterial cell walls (Wang *et al* 2016), or membrane proteins (Becker-Baldus *et al* 2015), with unprecedented sensitivity. Using this approach on microalgae should improve our knowledge of the complex entanglement of proteins, lipids and carbohydrates in cellular external matrices. It is exciting to see that, while in-cell SS-NMR is still the source of many new developments, it has now reached the maturity to be applied to whole microalgae to give better understanding of living organisms at an atomic level.

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