

Solid-State and Diffusion NMR Spectroscopy as Applied to Characterization of Complex Formulated Products and Biological Materials

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ABSTRACT

Solid-state and diffusion NMR spectroscopy are versatile tools that allow for studies of both structure and dynamics of complex solid and semisolid materials. We have developed a suit of pulse sequences based on polarization transfer that is readily applied to studies of formulations and their interactions with biological systems. In the present paper, we illustrate its applicability by using an incompletely hydrated surfactant system (tetradecylmaltoside, C₁₄G₂) and skin (stratum corneum, SC) as examples. In the former case, solid-state NMR has allowed us to study the dynamics of the system at the level of individual functional groups, and also to derive the surprisingly complex C₁₄G₂-H₂O phase diagram at water contents <5%. For SC, NMR data has allowed us to study the effect of natural moisturizing factor (NMF) on the dynamics of its lipid and protein constituents. The results challenge the conventional view that NMF protects the skin from dryness by increasing its water content.

Keywords: NMR, formulated products, skin, stratum corneum, phase diagram, glass transition

1 INTRODUCTION

Solid and semisolid formulations in pharmaceuticals, cosmetics and foods normally comprise complex structures on the molecular and colloidal length scales. Proper understanding of the functionality of a formulated product, as well as rational optimization of its physical and chemical stability, requires detailed understanding not only of the structure itself, but also of dynamics.

Solid-state NMR is a versatile tool for studies of complex colloidal systems. Over the last couple of years, we have systematically investigated the applicability of the method in the fields of formulated products and biological materials. The suit of pulse sequences that we have developed for this purpose is based on polarization transfer, and combines features from high-resolution NMR and solid-state NMR [1]. It allows for studies of both structure (in terms of the *orientational order parameter*) and dynamics (in terms of *re-orientational correlation times*) of solid and semisolid materials in a way that makes it directly

applicable to formulation design, prediction of product stability and understanding of interactions with biological tissue.

This paper briefly describes the principles of polarization transfer solid-state NMR, and illustrates how this methodology can be applied in formulation science and technology. Examples are extracted from our extensive work on lipid- and carbohydrate-based systems. More specifically, we use the sugar-based surfactant tetradecylmaltoside to illustrate how NMR allows for the structure and dynamics of a complex semisolid system to be studied with atomic resolution as a function of water content and temperature [2]. In order to illustrate that the same technique is directly applicable also to biological systems, we summarize some pertinent results on the properties of stratum corneum, and the way these properties depend on natural moisturizing factor [3]. These examples are used to show that the characteristics of phase transitions (e.g. glass transitions and chain melting in composite lipid systems) can be pinpointed in molecular detail. For instance, NMR can be used to elucidate the order by which a given molecule or molecular fragment “melts” (i.e. become mobile, as defined by the correlation time) when a heterogeneous material is heated, hydrated or subjected to other physical changes. This, in turn, allows the formulation scientist to understand the nature and practical implications of these phase transitions in more detail than do conventional techniques (for instance, calorimetry).

2 PRINCIPLES OF POLARIZATION TRANSFER NMR

The suit of pulse sequences used in the present work is based on polarization transfer [1], and is used to collect natural-abundance ¹³C NMR spectra. The three pulse sequences are *direct polarization* (DP), *ramped cross-polarization* (CP) [4], and *refocused insensitive nuclei enhanced by polarization transfer* (INEPT) [5]. For a given sample, the NMR data collected by the three individual pulse sequences provides a detailed map of the structure and dynamics of the sample on the molecular level. The DP experiment gives a spectrum containing signals from all ¹³C in the sample. The CP and INEPT experiments, on the other hand, discriminate between ¹³C nuclei situated in rigid and

mobile molecular moieties, respectively. In other words, signals in the CP spectrum originate from nuclei that are situated in a rigid local environment, whereas nuclei in a mobile local environment are silent. The opposite holds true for INEPT, which is sensitive only to nuclei in mobile groups. This discriminatory effect can be understood in more depth by referring to the *CH-bond orientational order parameter* (S_{CH}) and the *CH-bond re-orientational correlation time* (τ_{CH}). Here, S_{CH} provides a means to quantify the degree of order in which a given nuclei resides (e.g. to differentiate between crystalline versus amorphous regions in a structure), whereas τ_{CH} provides a quantitative description of the mobility of said nuclei ("solid" versus "liquid"). An isotropic liquid is characterized by low order (i.e. low value of S_{CH}) and high mobility (short τ_{CH}), and consequently gives signals only when using the INEPT pulse sequence. Crystalline materials, on the other hand, are characterized by high order (high S_{CH}) and low mobility (long τ_{CH}), which gives rise to signals only in the DP protocol. Anisotropic systems with high mobility represent an intermediate regime, which gives signal in both INEPT and CP, whereas nuclei with τ_{CH} in the microsecond regime are silent in both cases.

In conclusion, a detailed analysis of the NMR data obtained by DP, CP and INEPT makes it possible for us to paint a picture of the structural and dynamic state of a sample, with a resolution that allows for study of individual functional groups in a molecule.

3 EXPERIMENTAL

Samples were contained in hermetically sealed rotor inserts, which were placed in 4 mm magic angle spinning (MAS) rotors during measurements. Spectra were collected on a Bruker Avance-II 500 spectrometer operating at a ^{13}C frequency of 125 MHz. The instrument was equipped with a 4 mm CP/MAS probe, and the sample was subjected to magic-angle spinning at 5 kHz during acquisition. CP, DP and INEPT spectra were recorded using 68 kHz TPPM ^1H decoupling. Sample temperature was adjusted and maintained with a BVT-2000 temperature control unit.

The different solid phases of tetradecylmaltoside (C_{14}G_2) were prepared as previously described by Ericsson et al [6]. Prior to measurements, the phases were equilibrated in desiccators, in which the water activity was held constant by means of relevant saturated salt solutions. Water content was determined gravimetrically.

Skin tissue from porcine ears was dermatomed and placed on filter paper soaked in PBS solution with 0.2 wt% trypsin at 4 °C overnight to separate the stratum corneum (SC) [3]. The SC samples were dried and ground with a pestle and mortar. Lipid extracted SC (corneocytes) was prepared by extraction in chloroform [3]. In studies involving components in natural moisturizing factor (NMF), samples of SC or corneocytes were equilibrated with known amount of the component under study (urea, glycerol, PCA and UCA) under controlled water activity

(80% RH). NMR experiments on SC were conducted at 32 °C.

4 GLASS TRANSITIONS AND PHASE BEHAVIOR

As has been described in detail elsewhere [2,6], the phase behavior of C_{14}G_2 at low water content (<5% water) is very complicated and involves (at least) two crystalline anhydrides, a crystalline hemihydrate, a crystalline dihydrate, a liquid crystalline phase, a gel phase and a glass. All of these structures are lamellar, but differ in dynamics and structural details. The $\text{C}_{14}\text{G}_2\text{-H}_2\text{O}$ system at low water content therefore represents an excellent model system to verify the applicability of the NMR methodology in the characterization of complex solid and semisolid systems under "almost dry" conditions. In particular, the $\text{C}_{14}\text{G}_2\text{-H}_2\text{O}$ system can be used to show that NMR provides information that is not possible to extract using conventional structural and calorimetric techniques.

Determination of the glass transition temperature (T_g) is a case in point. Since a glass transition is not a first order phase transition, T_g is not a single, well-defined temperature, but rather a temperature range, over which the molecular mobility of the system increases continuously, rather than abruptly. Conventionally, T_g is determined by calorimetric means (normally by differential scanning calorimetry, DSC), and the glass transition is evident as a change in heat capacity (C_p). However, on the microscopic level, the glass transition manifests itself as a complex sequence of events in which the mobility of each functional group of the molecule gradually increases with temperature. This means, for instance, that at a given temperature, one functional group of a given molecule may be mobile, whereas another, adjacent group is still rigid. These events lend themselves to NMR studies, but are obviously not properly captured by techniques that measure bulk properties, such as calorimetry.

Our studies of the structure and dynamics of the disordered lamellar phase of C_{14}G_2 illustrate these points. The glass transition temperature of this system, as determined by DSC, has previously been reported to be 48 °C [6]. However, even 20 °C below this temperature, our NMR data show that the terminal parts of the alkyl chains (the C13 methylene and C14 methyl carbons) re-orient with τ_{CH} as low as nanoseconds. These groups are thus liquid-like at temperatures well below the glass transition temperature! Similarly, as the temperature is increased, the mobility of the methylene groups in the alkyl chain increases continuously in a manner that is dictated by the exact location of each methylene group in the alkyl chain. More specifically, at a given temperature within the glass transition range, τ_{CH} for the methylene groups close to the surfactant head-group may be orders of magnitude slower than that of the methyl terminal [7]. In a sense, the melting thus starts in the methyl terminal and propagates through the alkyl chain towards the head-group. Over a wide

temperature range, liquid-like and solid-like moieties consequently co-exist in the molecule.

The possibility to map glass transitions in a detailed manner and with molecular resolution has considerable practical consequences in the context of formulation science. Firstly, the physical and chemical stability of a “dry” formulated product (for instance, freeze-dried materials and solid pharmaceutical dosage such as tablets and capsules) is often directly dependent of the mobility of the individual components. Determination of the molecular mobility, rather than extrapolation from indirect bulk properties, is therefore extremely valuable for correct prediction of stability and shelf life under a given set of conditions (temperature, moisture). Secondly, the tactile and sensory properties of materials are also directly dependent on the molecular state (liquid versus solid) of the ingredients. This aspect is more relevant for cosmetics, where various types of waxes, as used in creams and lotions, illustrate the point that consumer appreciation is directly linked to the exact molecular state of key ingredients.

The studies of the glass transition in C₁₄G₂ primarily deal with the dynamics of the system, since the phase structure is retained during the process. However, the polarization-transfer NMR methodology is equally valuable in studies where the structure is the main aspect of interest, and, in particular, where structure and dynamics are both of critical importance for the complete understanding of the system. We illustrate this with our studies of the C₁₄G₂-H₂O system at low (<5%) water content. Generally speaking, studies of phase behavior under “almost dry” conditions are challenging in terms of both reproducible sample preparation and reliable characterization. Nevertheless, understanding of this part of the phase diagram is critical for (nominally) dry formulations, since moisture-induced phase transitions are generally detrimental to both stability and performance.

Our work on the C₁₄G₂-H₂O system shows that the NMR protocols (sample preparation, loading of sample cells and measurements) allow for detailed mapping of the phase behavior of complicated systems at low water content, and that the procedures are robust and possible to perform in a standard lab environment without dedicated preparative equipment. Furthermore, the suit of pulse sequences has allowed us to determine the exact relationship between the two anhydrous crystalline phases, the two crystalline hydrates and the liquid phases with which the solid phases co-exist above their respective melting points [2].

5 STRATUM CORNEUM AND NATURAL MOISTURIZING FACTOR

Stratum corneum (SC) is a lipid-protein membrane that constitutes the outermost layer of skin. The obvious biological function of SC is to provide an efficient barrier between the organism and the environment. In order to

maintain this vital function in an ever-changing environment, SC has to be able to cope with the quite substantial osmotic stress that is induced by changes in ambient temperature and water activity. To this end, the lipid-protein fraction of native SC is interacting with *natural moisturizing factor* (NMF), which is a mixture of small, mainly polar molecules that can act as osmolytes. The components in NMF include urea, glycerol, pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA), the first two of which are also used in various formulated products designed to moisturize dry skin.

It has long been known that proper hydration is essential for SC integrity [8], and the biophysical function of NMF is widely believed to hinge on the “water-retaining capacity” of its components. In other words, the presence of NMF is assumed to increase the water content in SC, hence protecting it from drying. In order to test this hypothesis, and to build more detailed understanding of the structure and dynamics of SC under various conditions, we subjected SC and SC-NMF mixtures to solid-state NMR analysis, using the suit of pulse sequences described above [3,9].

The lipid and protein fractions of SC were found to give characteristic signals that are separated from each other in the ¹³C NMR spectrum [9]. The protein fraction consists mainly of keratin filaments and gives signals in the range 40-70 ppm (Gly and Ser residues), whereas the signals from the alkyl chains of the lipids (primarily ceramides) are found at 15-35 ppm. Consequently, the properties of the two fractions can be studied separately in a single experiment.

In SC equilibrated at 80%RH, comparisons of CP and INEPT spectra show both the lipid and protein fractions to be predominantly rigid. The spectra also reveal that the alkyl chains of the lipids are present in both the all-trans conformation typical of crystalline materials, and in disordered distributions of trans- and gauche conformations. However, the INEPT spectra also suggest that a small fraction of methylene and methyl carbons undergo fast rotation around the C-C bond. Increasing the hydration level of SC by equilibration at 96%RH has a profound effect on the fluidity of the lipid and protein fractions. In this case, the INEPT signal from methyl and methylene group in the lipid fraction increases in intensity, as do the INEPT signals from terminal groups in the proteins. These features clearly substantiate the notion that an increased level of hydration promotes molecular mobility and fluidity in SC lipid and protein components. Nevertheless, the spectra also clearly suggest that the majority of the protein carbons are still residing in an environment that is rigid.

Interestingly, addition of 20 wt-% of urea to SC at 80%RH was found to have the same effect on the fluidity of the system as an increase of RH to 96% in the absence of urea. Just as for the system equilibrated at 96%RH, the urea-containing sample at 80%RH displays increased intensity of the INEPT signals from terminal protein groups and lipid alkyl chains. However, the presence of urea in SC

was found to have only marginal effects on the water content at 80%RH. We thus find strong evidence that components in NMF, by themselves, can have the same effect as water on SC fluidity. This conclusion is substantiated by the results from studies of the effects of glycerol and UCA on SC fluidity. In both cases, the compounds are found to increase the mobility of both the lipid and protein fractions in a way that resembles the effect of water. In addition, we find evidence that UCA, in particular, affects not only the mobility of the lipid alkyl chains, but also that of the headgroups. In contrast, the fourth NMF component, PCA, does not give rise to appreciable effects on SC properties at 80%RH. The absence of INEPT signals from UCL itself suggests that the lack of effect on SC fluidity is due to PCA being in the solid state under these conditions, which is also in agreement with calorimetric data.

The main conclusion from the studies is thus that at constant water activity, components in NMF increase the fluidity of both the protein and lipid fractions of SC, without inducing any appreciable effect on the SC water content. In other words, NMF components act as plasticizers in SC, just as water does. From a biophysical perspective, the presence of NMF can therefore be understood as a way to maintain SC integrity under the osmotic stress induced by dry conditions. The vapor pressure of water is high, which leads to a quick drying when the ambient water activity decreases. The vapor pressure of the NMF components, on the other hand, is low and these components are therefore retained in SC to a much higher extent, even under challenging conditions.

6 CONCLUSIONS

The results we have obtained on complex, partially hydrated systems of both natural and synthetic origin show that solid-state NMR provides structural and dynamic results on the intramolecular level, and that these results are of direct relevance for formulation science. The methodology is applicable both in the elucidation of interactions between formulated products and the biological system, and in product design and prediction of product stability.

REFERENCES

- [1] A. Nowacka, P. C. Mohr, J. Norrman, R. W. Martin, and D. Topgaard, *Langmuir*, 26, 16848, 2010.
- [2] S. Gustavsson, J. Algotsson, A. Nowacka, J. Reimer, S. Ulvenlund, and D. Topgaard, *Phys. Chem. Chem. Phys.*, submitted.
- [3] S. Björklund, J. M. Andersson, Q. D. Pham, A. Nowacka, and D. Topgaard, E. Sparr, *Soft Matter*, 10, 4535, 2014.
- [4] A. Pines, J. S. Waugh, and M. G. Gibby, *J. Phys. Chem.*, 56, 1776, 1972.
- [5] G. A. Morris, and R. Freeman, *J. Am. Chem. Soc.*, 101, 760, 1979.
- [6] C. A. Ericsson, L. C. Ericsson, V. Kocherbitov, O. Söderman, and S. Ulvenlund, *Phys. Chem. Chem. Phys.*, 7, 2970, 2005.
- [7] A. Nowacka, N. A. Bongartz, O. H. S. Ollila, T. Nylander, D. Topgaard, *J. Magn. Reson.* 230, 165, 2013.
- [8] I. H. Blank, *J. Invest. Dermatol.*, 21, 259, 1953.
- [9] S. Björklund, A. Nowacka, J. Bouwstra, E. Sparr, D. Topgaard, *PLoS ONE*, 8, e61889, 2013.