Improving Resolution In NMR In The Presence of Microcarriers Using Holmium-DOTA

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***Abstract*—This research is part of a larger project exploring the use of surface modified microcarriers, specifically polystyrene and PDMS, as a chromatographic medium to enable high resolution liquid state NMR-analysis of biological samples such as blood and plasma. The purpose of the microcarriers is to bind proteins and lipids, which distorts the NMR-spectrum, hence enabling identification of metabolites. This would enable for direct NMR-analysis of biological samples which would reduce procedure complexity, operation duration from several hours to a couple of minutes and finally usage and waste of organic solvents. The primary objective is to determine whether adding a paramagnetic solvent to a suspension of water and microcarriers can achieve high resolution NMR-peaks, through so called magnetic homogeneity. Experiments were conducted at the Biomedical Center, Lund University, where a NMR tube was filled with a microcarrier-water suspension and small amounts of fumarate, used to measure NMR peak linewidth, as a measure of resolution. To achieve homogeneity a solution of holmium DOTA, Ho-DOTA, was incrementally added to the tube. After each addition, the sample was mixed thoroughly and NMR analysis was performed to measure linewidth changes. The results suggest that adding Ho-DOTA can significantly enhance magnetic homogeneity and NMR resolution. In the PDMS suspension a minimum linewidth of 22Hz was obtained in proton-NMR and 8Hz in ^{13}C NMR spectroscopy. This finding holds potential for streamlining the analysis of complex biological samples, reducing the time and complexity involved in traditional NMR preparation methods. However, in the polystyrene solution line broadening was only achieved. Althought, this results suggest that our method might also be used to measure the magnetic susceptibility of an unknown material.**

I. Introduction

OUR project is interdisciplinary in nature, which means that we need to provide the necessary information from each of the fields that we use to help in understanding this complex subject.

A. Nuclear magnetic resonance (NMR)

NMR spectroscopy, is a spectroscopic technique used to analyze the constituents of compounds within a solution. It is widely used for research on chemical compounds and biological samples. It is based upon a few fundamental principles that we will now explain more in depth. Liquid state NMR is more useful for biological applications. Solid state NMR also exists.

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B. Physics

NMR is built upon the precession, or wobble, of a magnetic moment in certain atoms, when placed in a static magnetic field, as well as their interaction with a radio frequency(RF)-pulse. There are several atoms that have a certain precession when in a magnetic field, but in this report we will only focus on hydrogen NMR, also known as proton NMR. Even though NMR can be applied on other elements such as carbon, hydrogen is the most abundant element in biological samples and therefore best for our field of application.

Nuclear magnetic moment arises in atoms with an odd number of protons or neutrons and is a result of their intrinsic nuclear spin. One could think of nuclear spin as how the earth rotates around its own axis, while magnetic moment is the result of this spin. The magnetic moment is a vector and therefore has a magnitude and an orientation, see Figure 1. Each element which has magnetic moment, also has a element specific gyromagnetic ratio which impacts the Larmor frequency, which will explained in next section1.

Magnetization occurs when a static magnetic field, B0, is applied in the longitudinal direction, z, the general magnetic moment of the affected nucleus will align in the direction of the static field. This alignment will result in a net magnetization, which is the sum of all the individual magnetic moments pointing more towards one specific direction, in this case z. Without the magnetic field, each magnetic moment from each and every nuclei will have a random orientation, and hence cancels each other's magnetic moment. A second feature of the magnetic field is that each nuclei present in the field will exert a magnetic precession at a specific frequency. The precession is the magnetic moment, of a proton, which wobbles around its own axis aligned with the direction of the B0. The frequency of this precession is known as Larmor frequency and is specific for a given element in a given B0.

Radio frequency(RF)-pulse. After a static magnetization has been applied to a sample, we emit a radio frequency pulse. The frequency of this pulse is the same frequency as the Larmor frequency. The Larmor frequency, of 500Mhz, is the resonance frequency of all the protons in a magnetic field with a strength of 11.7T. When the protons are exposed to this RF-pulse they will excite resulting in a 90 degree flip of the net magnetization, from the z direction, to the transverse plane, also known as the xy plane.

Proton density. After the protons have been excited they will align with the xy plane sending out a small burst of energy which is picked up by a coil in the NMR spectrometer. This burst of energy is directly proportional with the proton density

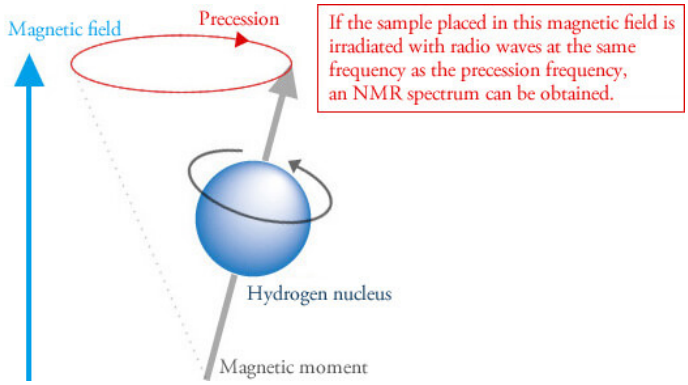

Figure 1. This picture is a visualization of the nuclear spin, magnetic moment, precession and the static magnetic field B0 and how to are related to each other. Reproduced from [1]

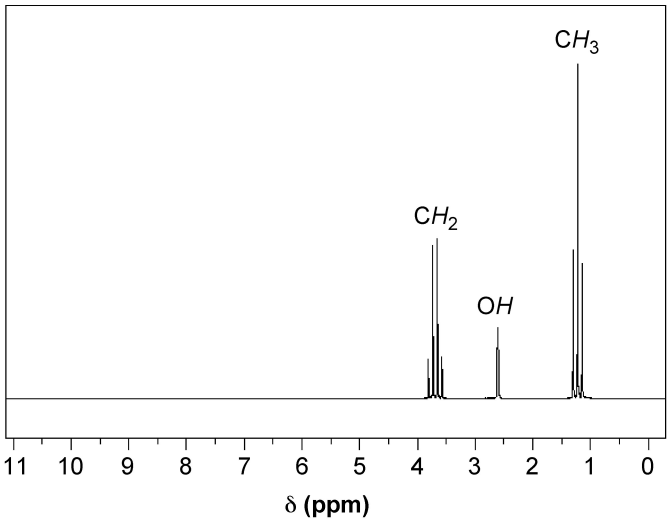

Figure 2. The spectrum shows a NMR-analysis of ethanol. Three different peaks, each corresponding to a specific group within the ethanol that has a bound hydrogen. Signal intensity is presented by the y-axis and chemical shift on the x- axis reproduced from [12].

in the sample.

Chemical shift is the variation in nuclear magnetic resonance frequency for a proton, caused by variations in electron distribution as a consequence of surrounding elements. It is measured as a relative deviation from the Larmor frequency and is usually presented in parts per million(ppm). Chemical shift makes it possible to identify the different protons within a molecule, since all hydrogen do not possess the same resonance frequency due to dissimilar chemical environments.

Transverse Relaxation, T2 is the rate at which the transversal net magnetization, after RF-pulse excitation, dephases. Immediately following the RF pulse, the magnetic moments of all protons will align in the same direction, but they will soon diverge and point in different directions. If T2 is short it refers to a fast diffusion rate, leading to a faster loss of net magnetization in the transversal plane. If T2 is long its the opposite, slow diffusion rate and therefore slower loss of net magnetization in the transversal plane.

The NMR spectrum When a NMR spectrum is obtained it displays the presence of certain groups within a molecule. All groups bound to hydrogen will be expressed as a peak in the spectrum. Each with a different chemical shift. By identifying peaks and or combination of peaks, the compounds in a sample can be determined [15]. This is shown in Figure 2.

C. Magnetic susceptibility and magnetic homogeneity

Magnetic susceptibility is a material specific property that describes the extent to which it will become magnetized in response to an external magnetic field. It directly influences how much the material will align with, or oppose, this field. All materials have different magnetic susceptibility and will hence align differently with the magnetic field. Paramagnetic material, such as Holmium, has a positive magnetic susceptibility and will align in the direction of the field while diamagnetic materials, such as water, PDMS(Polydimethylsiloxane) and polystyrene, have a negative susceptibility and will instead slightly oppose the external field [2]. In order to obtain a high quality NMR signal with narrow linewidth, the sample must be magnetically homogeneous. Magnetic homogeneity refers to a continuous uniform magnetic field across the whole sample. Mismatches in magnetic susceptibility between constituents within the sample can result in a magnetic inhomogeneous sample which leads to poor signal quality and overlapping peaks. In our case, in order to achieve homogeneity, the susceptibility of the solution must be matched with the magnetic susceptibility of the microcarriers [10].

D. Linewidth and resolution

Linewidth is defined as measuring the width of a signal peak at half-height. Signal quality, or resolution, is highly dependent on linewidth. If the resolution is bad the linewidth will be broad, leading to peaks overlapping. This leads to difficulties in differentiating, or identifying, different compounds within a sample. Linewidth is measured in delta Hz, since it is a difference between two points in the chemical shift [16].

E. Microcarriers

Microcarriers are very small particles, most often in the micro scale, made of some kind of polymer. In this project we will be looking at polystyrene and PDMS microcarriers, figure 3. These two materials have very similar magnetic susceptiblities, PDMS has -8.1 and polystyrene has around -8 [20] [9]. They are also suitable for use in biological samples because they are biocompatible and they adsorb proteins, separating them from the sample and allowing for good signal quality [11] [5].

F. Holmium

Holmium is a strong paramagnetic material with a volumetric magnetic susceptibility of 29535 ppm [3]. It was chosen for this project because Holmium salts are fully miscible in water, it does not oxidize metabolites in biological sample and it does not impact the pH and the T2 of our solution.

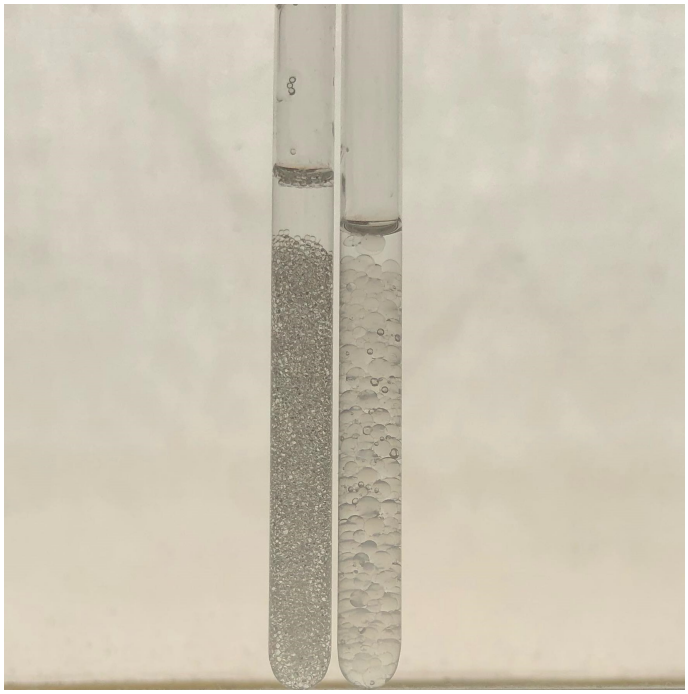

Figure 3. This is how the microcarriers looked when packed into the NMR tube, Polystyrene to the left and PDMS to the right

G. Problem

In the past decades, the field of analyzing metabolites within biological samples, such as blood plasma, has grown rapidly. Metabolite identification has been promising in finding biomarkers for diagnosing diseases but also understanding metabolic pathways [6], [13]. However, one of the main issues with NMR analysis of biological samples is the complexity of the sample consisting of large molecules such as proteins. The presence of larger molecules can lead to very broad peaks making it difficult to detect the metabolites of interest. There exist a couple of techniques today that can improve the signal quality and reduce overlapping, one of these is filtering of proteins and lipids. However, filtration of these molecules takes several hours and consists of several steps [5]. Therefore, there is a great need to reduce time and complexity both in order to facilitate research and development(R and D) but also to make it more clinical viable towards diagnostics. Hopefully preparation can be done faster by adding a chromatographic medium, such as surface modified polystyrene or PDMS microcarriers, that can adsorb the proteins to their surface [11]. However, adding particles to a solution effects the magnetic susceptibility of the solution causing line broadening and hence bad resolution. [8]

H. Agenda

This report has focused on whether signal quality, with regards to linewidth, can be improved by achieving magnetic homogeneity using a solvent, after microcarriers have been added to the solution. In the method and material section we first describe what solvents and machines were used, then we describe our calculations and set up and lastly we describe the experiment and discuss the results.

II. Materials and methods

A. Materials

The stock solution was made of Holmium Chloride hexahydrate, water, and tetraxetan (DOTA). We used two different types of microcarriers, polystyrene and PDMS, and suspended them both respectively in H_2O and fumarate. A 600μL NMR tube, pretreated with a 2% Hellmanex solution, was used. Titration was performed with a pipette. NMR analysis was done in an 11.7 Tesla NMR magnet. Data acquisition was done using the program VNMR. The values used for the different materials susceptibilities can be found in table I

Table I
Volumetric magnetic susceptibility of some relevant materials received from literature, [3], [20], [9], [7]

Material	Volumetric Magnetic Susceptibility, κ (SI, ppm)
Holmium	29535
H_2O	-9.03
Polystyrene	-8.0
PDMS	-8.1

B. Calculating desired Holmium-DOTA concentration

In this section we calculate the desired concentration of Holmium-DOTA needed to reach magnetic homogeneity.

$$\kappa_{\text{Mix}} = \kappa_{\text{Ho}} x + (1-x)\kappa_{\text{H2O}} \tag{1}$$

Volumetric magnetic susceptibility of the solution, κ_{mix}, calculated by the magnetic susceptibility of the additive, κ_{Ho}, and the water, κ_{H2O}. x is the volume fraction of the additive, in this case Holmium, in the entire solution [8].

If the susceptibility of the solution is to be matched with the polystyrene and PDMS particles, an expression derived from Formula 1 can be used. The volume fraction of the additive, which is needed to achieve magnetic homogeneity, can thus be determined with the following relationship.

$$x = \frac{\kappa_{\text{Polystyrene}} - \kappa_{\text{H2O}}}{\kappa_{\text{Ho}} - \kappa_{\text{H2O}}} \tag{2}$$

Volume fraction, x, of the additive calculated based on susceptibility of compounds within the sample solution

$$C = \frac{n_{\text{Ho}}}{V_{\text{Total}}} \quad n = \frac{m}{M_{\text{Ho}}} \quad m = \rho_{\text{Ho}} \cdot V_{\text{Ho}} \tag{3}$$

Combining the relations above, using substitution, a new relation for the concentration, C, of the additive needed to achieve magnetic homogeneity can be derived.

$$C = \frac{V_{\text{Ho}}}{V_{\text{Total}}} \cdot \frac{\rho}{M} \Leftrightarrow C = x \cdot \frac{\rho}{M} \tag{4}$$

Desired concentration calculated from volume fraction, the density of the additive and molar mass, M, of the additive.

C. Laboratory

The first step of the laboratory procedure was to prepare a stock solution of 5 mM Ho-DOTA. This was done by first mixing holmium chloride hexahydrate with water and then adding DOTA(1:1 ratio). The next step was to suspend the microcarriers in water and add the suspension to the 600μL NMR tube. Excess water on top of the beads, in the tube, was removed using a glass pipette. To establish out the amount of water in the tube we filled another 600ul NMR tube with just water and compared the water height with the microcarrier tube. Randomly packed same sized spheres have a void fraction ranging from 36-40% depending on whether they are loosely or tightly packed [17]. We assumed a loose pack and used 40% as void fraction to estimate the solution volume in the NMR tube. The estimated volume value was used to calculate the amount of Ho-DOTA solution to titrate, in order to keep track of the concentration within the tube. Ho-DOTA was incrementally added and the linewidth for fumarate and water was measured, after NMR analysis, for each addition of the stock solution. This method was used for two different microcarriers. First we used polystyrene, then we used PDMS.

III. Results

Table II
ESTIMATED VALUES TO ACHIEVE MAGNETIC HOMOGENEITY

Material	Volume fraction, x	Concentration (mM)
PDMS	0.0000315	1.68
Polystyrene	0.0000349	1.86

The values in Table II were obtained using the calculations earlier provided together with the values from Table I as well as the density, $\rho_{\text{Ho}} = 8800\,\text{g/dm}^3$, and molar mass, $M_{\text{Ho}} = 164.93\,\text{g/mol}$, of Holmium.

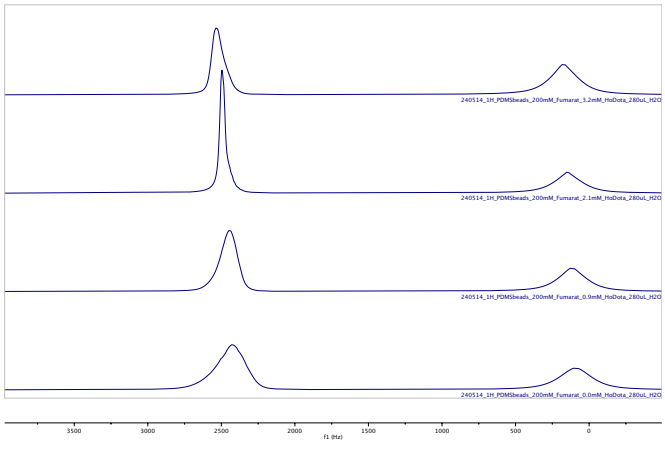

Figure 4. Multiple spectrums acquired from the analysis on Ho-DOTA in PDMS suspension. The peak on the left is the water peak and the peak on the right is the PDMS peak. Each of the spectrums are at different concentrations of Ho-DOTA

In Figure 4 we get a visual representation of how the linewidth changed depending on the concentration of

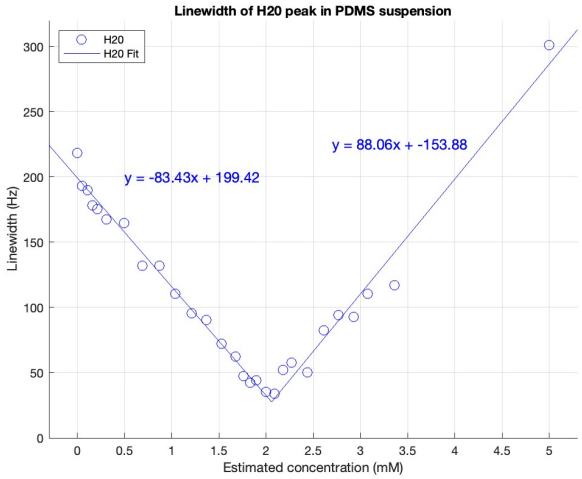

Figure 5. Datapoints of how linewidth, on the y-axis, of the H20 peak is affected by concentration of Ho(III), on the x-axis.

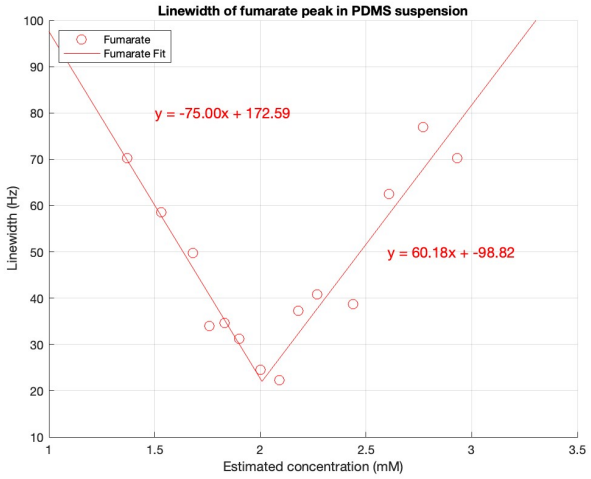

Figure 6. Datapoints of how linewidth, on the y-axis, of the fumarate peak is affected by concentration of Ho(III), on the x-axis.

holmium. In the bottom spectrtum Ho-DOTA is not present. The second spectrum from the bottom has 0.9mM Ho-DOTA. The third spectrum from the bottom, where minimum linewidth was reached, has 2.1 mM Ho-DOTA. The spectrum at the top has 3.2 mM Ho-DOTA.

The linewidth for fumarate and H_2O in PDMS suspension was measured simultaneously for a single sample. However, fumarate contains fewer data points due to H_2O signal overlapping the fumarate peak at broader linedwidths, making it impossible to accurately measure. This issue occurred around a linewidth of 100Hz. In Figure 5 and 6 the data set was split into two categories and linear regression was performed on each data sets. The linear relation for each fit is expressed within the figures. The split of the data set was based on where the lowest linewidth occurred. We see in the figures 5 and 6

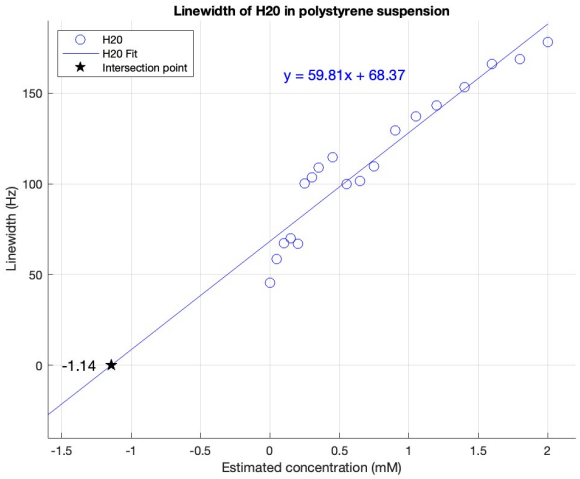

Figure 7. Datapoints of how linewidth, on the y-axis, of the H20 peak is affected concentration of Ho(III), on the x-axis.

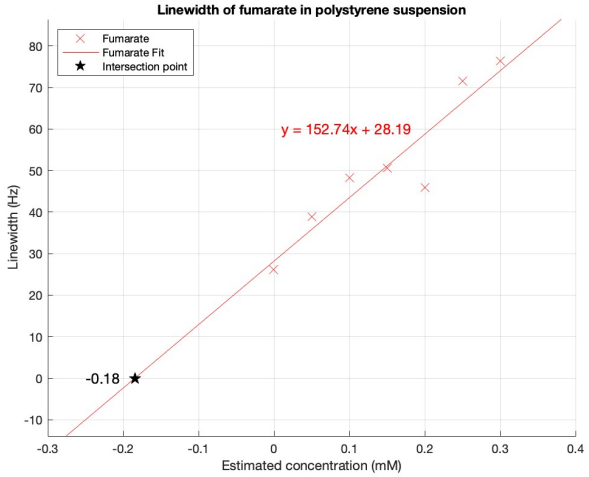

Figure 8. Datapoints of how linewidth, on the y-axis, of the fumarate peak is affected by concentration of Ho(III), on the x-axis.

that a minimum linewidth of 33.66 Hz for the water peak and 22.28Hz for the fumarate peak were achieved.

Figure 7 and Figure 8 are the results from our measurements done on polystyrene suspension. Linear regression was performed to fit a line based on data points. Furthermore, intersection point between the fitted line and the x-axis, x=0, is also marked.

IV. Discussion

The aim of our project was to increase NMR signal quality in the presence of microcarriers. Let us discuss now whether or not this is possible, what could have been done better and what the future may hold for this method.

A. Solvent selection

We started off by discussing several choices, DMSO, manganese chloride, acetic acid, holmium chloride and gadolinium dota. The decision on which solvent would be best came down to many different criterias. First of all the solvent should have a susceptibility which is higher than the water since PDMS has a higher magnetic susceptibility than waterI. The additive must also be fully miscible in water. Furthermore, the solvent should not be able to oxidize metabolites since they are the compounds of interest in NMR analysis of biological samples. The solvent can not impact the pH either because this could also ruin the metabolites [18]. Finally, the solvent may not affect the samples T2. If the solvent shortens T2 the constituents will diffuse too quickly during data acquisition which leads to poor signal intensity but also line broadening, essentially making the data useless [14]. Considering all these criterias we came to the conclusion that holmium chloride was the most suitable solvent.

The reason we used DOTA is because it is a chelator, or a complexing agent, used to bound and hold compounds. It is structurally similar to heme, as seen in Figure 9, the compound in blood which holds iron ions that in turn can bind oxygen [4]. In our case DOTA was added for the purpose of binding the holmium ion (Ho(III)).

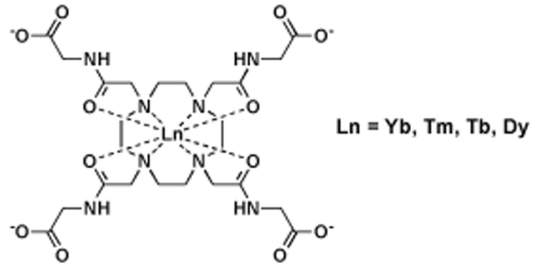

Figure 9. Illustration of the chemical structure of DOTA binding a lanthanide. Therefore, Ho(III) will bind to the center site of the compund. Reproduced from [19].

A short experiment was conducted to conclude the impact of DOTA on the linewidth. From the experiment we realised that using DOTA greatly reduced the linewidth. This can be explained by the increased distance between the holmium ion and the water after DOTA encapsulates the ions. DOTA is large complex compared to Ho(III), which explains the increased distance. This decreases the effect from the dipole-dipole interaction, between the paramagnetic and the water dipole, which is inversely proportional to the sixth power of distance(1/r6) [2]. Which means that a short distance increment drastically reduces the power of this interaction. Furthermore, the dipole-dipole interaction induces shortened T2 relaxation time which broadens the NMR signal. Therefore, by reduced dipole-dipole interaction NMR resolution is imporved [14] [2].

B. Polystyrene Suspension

As shown in Figure 7 and 8, the addition of Ho-DOTA did not shorten the linewidth, it only made it broader which was not expected. According to our calculations we were expecting

the linewidth to shorten until we reached a concentration of 1.6 mM Ho-DOTA, and from there the linewidth would broaden again. This might be because the microcarriers we ordered were not made up of 100% polystyrene, instead they could have been contaminated with something else which would have affected their magnetic susceptibility. Another reason could be the difficulty of mixing the suspension after holmium was added. Difficult mixing was caused by a small tube diameter, the tube being fragile and the beads acting as a plug in the tube when mixing, thus making it hard or us to be sure the solution was homogeneous. This could have lead to some regions of the sample having higher concentrations of Ho-DOTA than others. This could also explain the larger jumps between some of the data points in the result. However, using the intersection point, $= -1.14$, from Figure 7 we can calculate the magnetic susceptibility using the calculations in the methods backwards. This will give us a susceptibility of $\kappa = -9.66$. This value is interesting because it is very close to the magnetic susceptibility of polyethylene, $\kappa = -9.67$. What could have happened is that we received polyethylene instead of polystyrene. This statement could be supported by the results from the PDMS suspension which is more in line with literature suggestions. Although, looking at the intersection point for fumarate in polystyrene suspension, Figure 8, a somewhat different susceptibility, $\kappa = -8.93$, is obtained. However, the fitted line for fumarate is based on a smaller data set than the H2O line.

C. PDMS Suspension

The results from our PDMS suspension experiment were a lot more in line with what we had expected, however not completely. We had expected the linewidth to reach minimum width around 1.6 mM Ho-DOTA, but instead it was achieved at around 2.1mM. This is most likely due to the uncertainties of the amount of water the beads were suspended in from the start. Could also be consequence of some uncertainties regarding to the volumes titrated, since a 100μL pipette was used to add 10μL.

Nonetheless the result is promising and strengthens our hypothesis that a sample that has been mixed with microcarriers can still give a reasonable spectrum by mixing with a optimally designed additive compound, such as our Ho-DOTA solvent.

D. Potential use case for ^{13}C NMR spectroscopy

The minimum linewidth achieved was 22.3 Hz for fumarate, which is not a resolution good enough for analyzing metabolites with proton NMR, which requires a thin linewidth, less than 10Hz. However, this method instead might be applicable for ^{13}C NMR spectroscopy of metabolites. Since the distance between peaks in carbon NMR is greater making resolution less of an issue. Linewidth would also become 4 times thinner in ^{13}C spectroscopy because the gyromagnetic ratio of carbon is exactly 4 times higher than of a proton, which would theoretically yield a linewidth at 5.6 Hz. Such linewidth is concidered great in ^{13}C NMR

We did a final ^{13}C NMR analysis with PDMS and Ho-DOTA and received this spectrum in Figure 10. The signal to noise ratio is good as well as the linewidth, at 8Hz, which goes to show that it is applicable to biological samples.

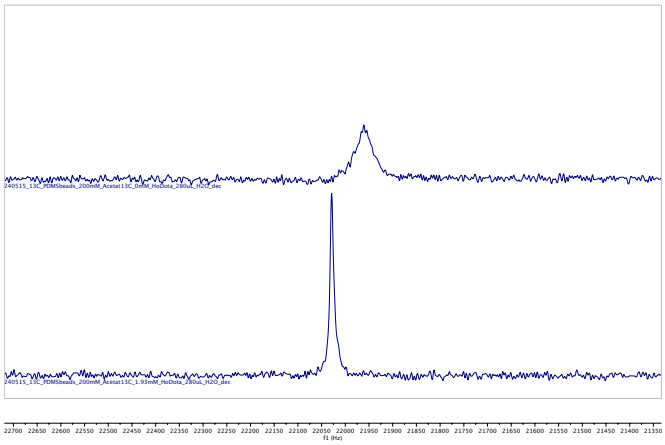

Figure 10. A ^{13}C spectrum done on acetate and PDMS. The first spectrum on top is with no Ho-Dota and the peak below is with 1.93 mM Ho-Dota.

E. Source of error and improvements

One source of error, shortly mentioned before, could be related to difficulties ensuring proper mixing after each addition of Holmium. Furthermore, considering the small stock volumes added to the larger suspension volume, it is hard to ensure proper distribution of the added stock. Another source of error was the difficulties of handling the microcarriers. Because the carriers are so small and the NMR tube is fragile and very thin it was not possible to know exactly how much water the microcarriers were suspended in, so we had to estimate. As stated in laboratory procedure the estimation was based on a void volume of 40%. This estimation might have been wrong due to the packing density but also because the microcarriers were not fully submerged. Therefore we might have had less water than expected.

F. Sustainable development and ethics

Delving into the realms of sustainability and ethics within NMR spectroscopy, we uncover several crucial considerations. Firstly, the sustainability aspect revolves around the environmental impact of NMR spectroscopy instrumentation and practices. Modern NMR instruments often require significant energy consumption and resources for manufacturing. However, efforts towards sustainability involve developing more energy-efficient instruments, implementing recycling programs for consumables, and reducing the use of hazardous chemicals in sample preparation.

On the ethical front, issues such as data integrity, responsible conduct of research, and equitable access to NMR facilities come into play. Researchers must adhere to ethical standards in data collection, analysis, and reporting to ensure the credibility and reproducibility of scientific findings. Moreover, promoting inclusivity and accessibility in NMR facilities is crucial for fostering a diverse and collaborative research community.

V. Conclusion

Our thesis is that we can shorten the linewidth of a sample with suspended microcarriers by adding a solvent of Ho-DOTA. This also holds the potential of using microcarriers as a chromatographic medium to separate proteins from biological samples to streamline sample preparation before NMR anaysis of metabolites. Despite the minimum obtained linewidth of around 20Hz for proton NMR was not small enough to be used for high resolution proton NMR of complex mixtures. The obtained ^{13}C-linewidth of 8Hz was very good which allows for use of microcarriers in ^{13}C NMR spectroscopy.

VI. Acknowledgements

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