Deuterium NMR and Intracellular Tumour pH Measurement

Can deuterium NMR be used as a suitable method for determining intracellular pH in tumour cells

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Abstract

The pH profile of the environment around a cancer site can provide information about factors such as cancer type and metabolism, which can aid diagnosis and treatment of cancer patients. Nuclear magnetic resonance, or NMR, is the leading principle behind MRI. Using NMR, chemical information about a sample can be obtained from its magnetic resonance properties. These properties may shift with pH level changes, and for this reason ^{1}H NMR has the potential to be a useful tool as it can give pH information quickly and non-invasively. However, since nearly all physiological compounds are abundant in ^{1}H, this technique suffers from high background signal levels. Using a deuterium (^{2}H or D) probe instead of a hydrogen (^{1}H) probe could provide a more specific measurement, as deuterium is not naturally abundant in the body and will therefore not produce much background signal. Histidine and imidazole were chosen as probe molecules and their rind carbon protons were exchanged with deuterium atoms. These deuterated probes were shown to have a strong shift in their ^{2}H NMR spectra in response to pH changes, and could therefore make for good pH probes in tumours. This confirms what could be expected from their behaviour in ^{1}H NMR. Histidine, being an amino acid, also offers the possibility of including it in a peptide, making it possible to tune the properties of the deuterated probe to the specific purpose of binding with the cells. A peptide synthesis was attempted but mass spectrometry showed it to be unsuccessful. Cell incubations were performed on HeLa cells but no probe signal could be measured, likely due to the probes not being pure. More research is needed on the toxicity of the probe molecules and their cell binding properties. The possibilities of creating a purpose-built peptide for this technique also warrant more research.

Contents

Introduction

Tumour pH

The acidity of the environment in and around a tumour site is greatly affected by the tumour and its characteristics. A tumour that grows and metabolises rapidly has a high excretion rate of acidic waste products. This causes acidic build-up around the tumour, which is often aggravated by poor perfusion, typical of cancer sites[1]. Despite being its own pathological symptom, this acidic build-up, or acidosis[2], could provide valuable information about a tumour's characteristics, including its metabolism, rate of growth, and possibly the type of tumour.

Acidosis is a consequence of the metabolism of glucose by tumour cells[3]. Tumour vasculature is erratic and inefficient. This can result in a low supply of O_2 to the tumour cells, causing the cells to metabolise glucose via the anaerobic route, see figure 1.1. Lactate is formed as a waste product and excreted from the cell. The same erratic vasculature also causes poor perfusion which aggravates build-up of this waste product.

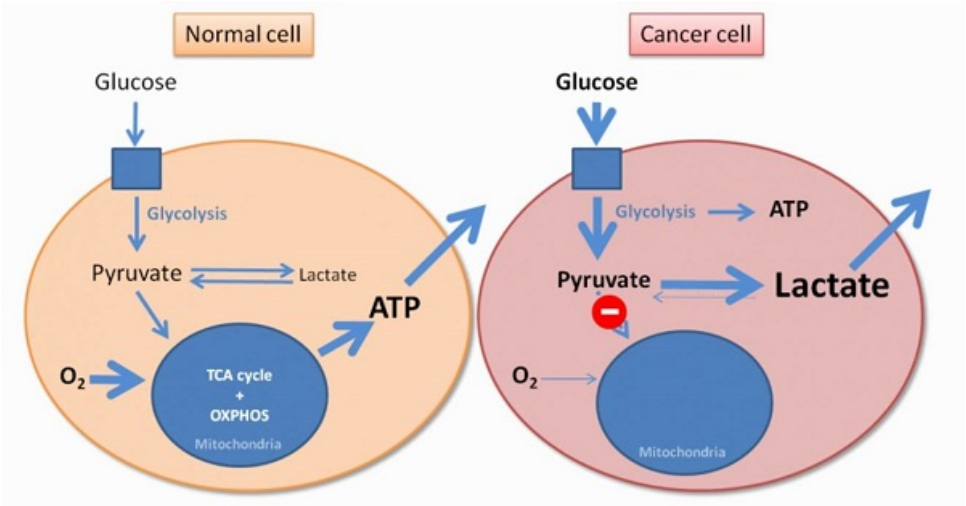

Figure 1.1: Lactate excretion by cancer cell[4]

The measurement of this pH can be done in a number of ways, including PET with pH sensitive tracers, optical techniques or MRI. However, measuring tumour pH is still an emerging clinical practice[1], and needs more development to be adopted on a large scale.

NMR

Nuclear magnetic resonance or NMR is an imaging technology that relies on the varying magnetic resonance properties of atoms in a molecule. It is the driving principle behind MRI, meaning that any gains made in pH measurement in NMR have the potential to be translated to a clinical setting in MRI. Measuring pH using MRI has the benefit of being non-invasive and could therefore be a valuable tool for diagnosis and monitoring of cancers. In 1998, Gasparovic *et al.*[5] used special probe molecules to reliably measure the pH in a tumour site using proton NMR, or ^{1}H NMR. The results were promising, and a great improvement in terms of signal to noise ratio and speed over the dominant method at the time, ^{31}P NMR. However, since this technique relies on hydrogen atoms for its signal, it can still suffer from high background noise. Hydrogen atoms are everywhere in the body, with nearly all biological compounds such as proteins, carbohydrates and metabolites containing a large number of hydrogen atoms.

A possible solution to this problem could be to use deuterium NMR. Deuterium, ^{2}H, or D, is an isotope of hydrogen that has a neutron in its core, as well as a proton, and is not naturally abundant in the body. Using a special probe, NMR can "look at" only ^{2}H atoms, which has very low background. Deuterium-containing probe molecules that enter the cells should be the only source of deuterium[6], and therefore the only signal in the ^{2}H NMR spectrum. This should greatly reduce background noise and could therefore improve the viability of MRI pH measurement.

Research Outline

In order to test the viability of deuterium NMR as a tumour pH measurement tool, a few steps needed to be completed.

A set of probes needed to be selected and deuterated so that they are visible in ^{2}H NMR. Next, the pH behaviour of these probes in NMR needed to be mapped. A titration was done to achieve this. Then, a cell culture was prepared and incubated with solutions of the different probes.

Finally, a pH measurement was attempted on the cells. This is done by seeing if there is a signal visible from the probe molecules. If so, it should be compared with the titration data. This then hopefully produces a pH value that is in accordance with an expected value from the literature.

2.1 Probe selection

The selected probes for this purpose are imidazole, histidine and a histidine-containing peptide. Probes need to have favourable pH characteristics and be likely to be taken up by cells. Favourable pH characteristics means that the probe NMR signal will change significantly, reliably and predictably with changing pH conditions.

2.1.1 Imidazole

Imidazole (shown in figure 2.1) is likely to be a good candidate for a pH probe because it has favourable pH characteristics and cell uptake[5]. Its pH sensitivity is due to it having two nitrogen positions that can be protonated or deprotonated, depending on the acidity of the environment. These two positions mean that there are three possible protonation states: fully protonated (in low pH), partially protonated (neutral pH) and fully deprotonated (high pH). This is shown in figure 2.2. This results in imidazole having two pK_a points: at 7.2 and 14.4 [8].

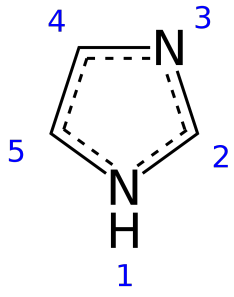

Figure 2.1: The imidazole molecule with atom numbering [7]

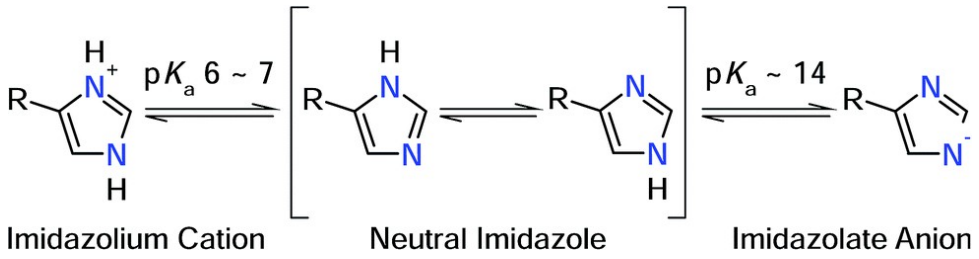

Figure 2.2: Deprotonation of imidazole and derivatives [9] with pK_a 1 = 7.2 and pK_a 2 = 14.4 [8]

2.1.2 Histidine

Histidine was chosen as a potential probe because it also has favourable pH behaviour and should be taken up by cells[5]. In addition to this, histidine is an amino acid. This means that it offers the possibility of creating a deuterated-histidine-containing peptide.

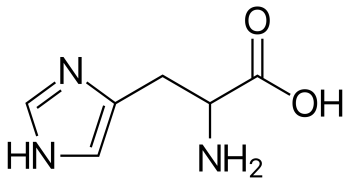

Figure 2.3: Histidine [10]

2.1.3 Peptide

A peptide that contains a deuterated histidine unit could have great benefits over regular histidine. Peptides can be synthesised in any configuration, meaning that their characteristics can be carefully chosen. Properties like cell uptake, toxicity, solubility and many others can be tuned to fit our purpose.

The peptide that was chosen is HAIYPRH[11]. It is a small, cell-penetrating peptide[12] with a histidine at both ends. Its size has the benefit of making it easy to synthesise and also aids cell uptake[13].

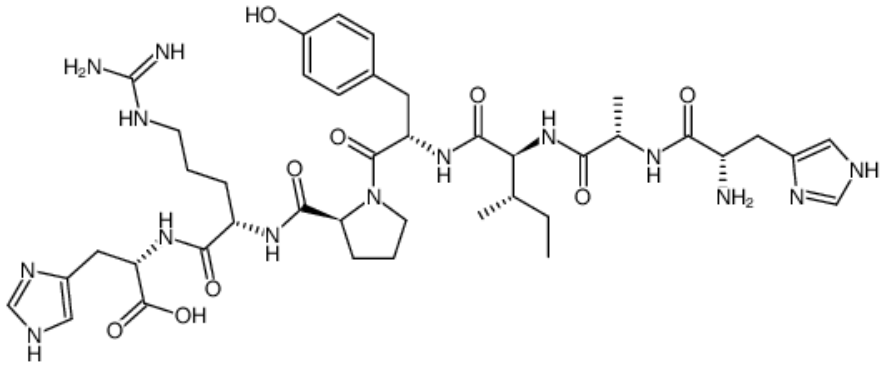

Figure 2.4: the HAIYPRH peptide[14]

2.1.4 Cell line selection

To conduct the cell research, a suitable cell line needs to be selected to perform the incubations on. HeLa cells were chosen for this purpose because of their high rate of endocytosis and high resilience [15]. This made it likely for the cells to survive the incubation process and take up the probes.

Methods

An overview of substances and their origin is provided in table 6.1.

3.1 Probe deuteration

In order to use the selected probes as a target for ^{2}H NMR, they need to be deuterated to some extent. This means that the H-atoms on C2 and C5 ring carbons should be replaced by deuterium atoms, as depicted in figure 3.1.

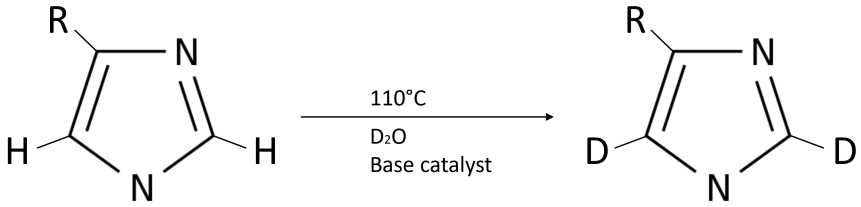

Figure 3.1: Deuteration of imidazole and derivatives

3.1.1 Imidazole

1250 mg of imidazole was added to a round bottom flask and boiled in heavy water (D_2O) for 3 nights in excess potassium hydroxide (KOH) as a catalyst. The addition of bases has been known to facilitate the H-D exchange on imidazole[16]. The process was done in a reflux setup with the oil bath temperature set to 110°C. The reaction mixture was continuously stirred using a magnetic stirrer, and the internal volume of the reflux was kept under argon.

Isolation of imidazole

In order to extract the imidazole from the mixture with D_2O and KOH, it was dried in the rotary evaporator and subsequently freeze-dried. It was then dissolved in tetrahydrofuran (THF), as imidazole dissolves in THF to some extent, whereas KOH does not[17]. Some magnesium sulphate was added to precipitate with any remaining D_2O in the mixture. The mixture was stirred heavily and the solid matter was separated from the THF-imidazole mixture using filter paper. The filtered liquid was then vaporised on a rotary evaporator, leaving behind the deuterated imidazole.

This process was first attempted using acetone, as it dissolves imidazole but not KOH[18]. However, this caused the product to turn a brownish red, probably because of unwanted reactions between the acetone and KOH.

3.1.2 Histidine

Histidine was first deuterated using the same process as imidazole above. However, it turned out to be too difficult to separate the KOH catalyst from the product with any available solvent. Therefore the process was repeated using triethylamine (TEA) as a base catalyst. TEA has the benefit of being liquid at room temperature, meaning that it can be evaporated instead of filtered.

1250 mg of Boc-l-histidine was deuterated by boiling in TEA. The reflux setup was the same as for imidazole. The process had to be run for 4 nights in order to get adequate deuteration. After this time, the TEA was evaporated away in the rotary evaporator. It is to be noted that on the 3rd day of deuteration, the reflux was discovered with some of the histidine built up in solid form on the side of the round bottom flask. This is discussed further in the Results section.

3.1.3 Deprotection of histidine

The protecting Boc group on the histidine was removed using the strong acid trifluoroacetic acid, or TFA[19]. To accomplish this, a test quantity of 50 mg of regular, undeuterated histidine was placed in a 25/75 solution of TFA/dichloromethane. This mixture was stirred for two hours at room temperature and then the liquids were removed on the rotary evaporator. An NMR spectrum was then taken of the product. It is compared below with a spectrum taken of untreated histidine (Figure 3.2). The peak closest to 1 ppm corresponds to the protecting group. Its integral value relative to that of the alpha carbon peak has changed from 7.3 to 0.093. This corresponds to a deprotection of 98.7%. These spectra therefore demonstrate that the deprotection method using TFA in DCM is effective.

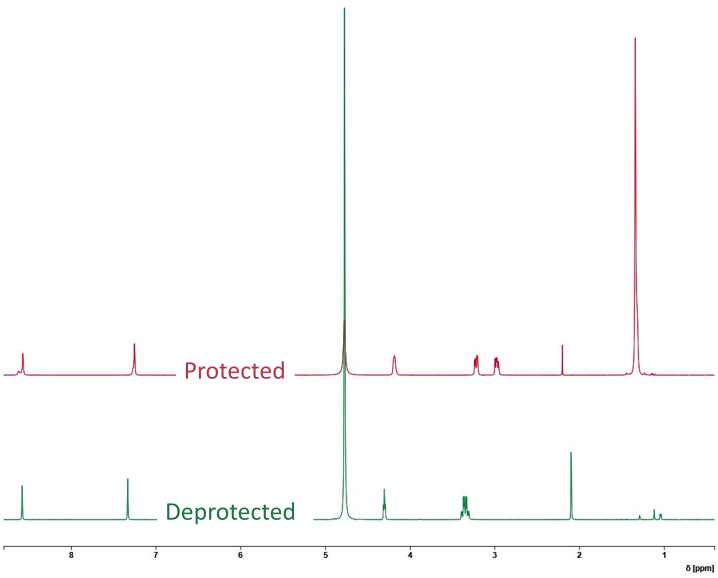

Figure 3.2: The deprotection of undeuterated histidine

The above result makes it worthwhile to repeat the process on 50 mg of deuterated histidine. The resulting spectrum is shown in figure 3.3.

Again, the Boc peak has almost fully disappeared after deprotection, with the integral value going from 0.56 to 0.056. Another peak at 2.1ppm can also be seen to disappear. This peak

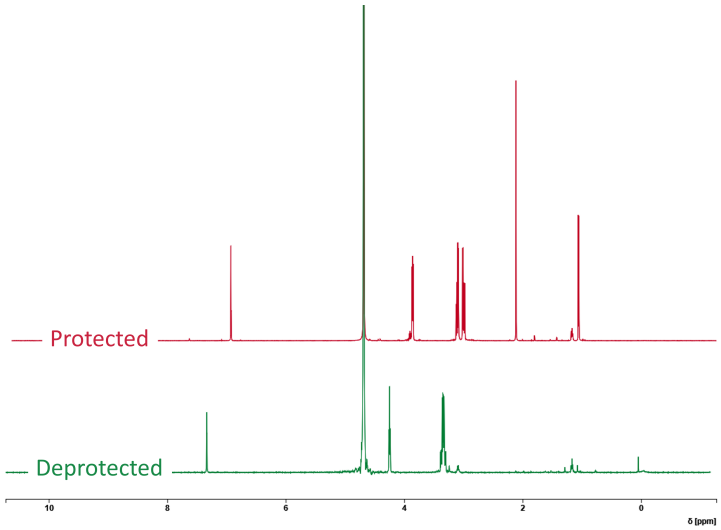

Figure 3.3: The deprotection of deuterated histidine

can likely be attributed to acetone residue from cleaning the tube, which was present in one experiment but not the other.

3.1.4 Peptide synthesis

To synthesise the peptide, a Bruker Smartflex solid support resin synthesiser was used. The peptide is synthesised by coupling the C-terminal amino acid to a resin and performing addition steps in the C - N sequence order. The peptide synthesiser can only add to Fmoc-protected amino acids. This means that the Boc-protected deuterated histidine must be added as the last step in the addition chain, as nothing can be added to it anymore. Furthermore, because of the amount of unknown variables surrounding the deuterated histidine, like possible racemisation, degradation, inhomogeneity etc, the decision was made to synthesise the first six units on the synthesiser, and add the deuterated histidine manually. If successful, the final product would then be $^{N}H_{D}AIYPRH^{C}$, with N and C indicating the two termini, and D indicating the deuterated compound.

Manual Addition

The manual addition of deuterated histidine is done in three steps: swelling, coupling and cleaving. The swelling step consists of swelling the condensed resin-coupled product from the automatic synthesis in N-Methyl-2-pyrrolidone (NMP) so that it is decondensed and reactive. Then, after removing the NMP, the coupling step is done with an NMP, Hydroxybenzotriazole (HOBT), Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU) and histidine solution, and stirring at room temperature for half an hour. Finally, the product is cleaved from the resin using a cleavage cocktail consisting of TFA, Titanium(II) Sulfide (TiS) and H_2O. The mixture is washed in cold diethylether with the peptide precipitating in the liquid.

Mass Spectrometry

In order to verify if the peptide was created, a MALDI mass spectrometer was used. A sample of the peptide synthesis product was dissolved in 50/49/1 acetonitrile/H_2O/TFA. 10μl of this solution was added to 10 μl of a saturated matrix solution of α-Cyano-4-hydroxycinnamic acid.

A 1 μl drop of this mixture was then transferred to the sample plate of the mass spectrometer, after which the measurement was done.

3.2 pH titration

In order to determine the pH in a sample from an NMR spectrum, we need to know the chemical shift of the D-atoms on the probe molecules as a function of the pH. This means that a titration needs to be done. A sample of each probe in a PBS buffered solution of physiological saline concentration will be analysed in the NMR for a range of pH values. The pH values range from 3 to 11 with more scans being done in the physiological range of 6 to 8. This data can then be used to find a chemical shift curve for a range of pH.

Solutions were created by dissolving a quantity of probe in PBS, and then transferring 500 μl of this solution to the NMR tube. The pH was then controlled using micropipet drops of 1M NaOH and 1M HCl solutions. An NMR tube compatible glass electrode was used to measure the pH in the tubes. For imidazole, the solution in the tube contained 10.06mg imidazole. For histidine, the exact concentration is not known, as the solution reached its limit of solubility before transferring to the NMR tube. The concentration must be lower than 10mg/0.5ml, and is probably significantly less. The pH of the imidazole solution as a function of the amount HCl added can be seen in figure 3.4. A similar behaviour was observed for deprotected histidine, but this data was not logged as accurately.

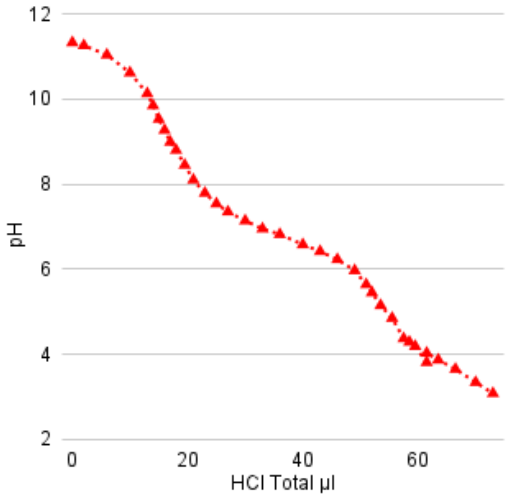

Figure 3.4: The pH of imidazole

Two tipping points are visible in the graph, which indicate two pK_a points for the compound. This is due to imidazole having two nitrogen positions that can be protonated or deprotonated, as explained in section 2.1.1. The titration starts at a high pH, presumably because some of the catalyst base was still present.

3.2.1 NMR Measurements and Processing

All NMR measurements were done on a 600MHz, 14.1T Bruker system. Spectra were processed in Bruker Topspin and exported to MATLAB or nmruim.org for visualisation. Phase corrections, calibrations and integral measurements were also done in Topspin. For ^{1}H measurements, the number of scans was always 4, with the spectral width 11.9015 ppm. For ^{2}H measurements, the number of scans was 32 unless specified otherwise, with a spectral width of 20.1007 ppm.

3.2.2 Cell incubation

To grow the HeLa cells, a population was thawed and cultured in Dulbecco's Modified Eagle Medium (DMEM). 3 flasks were prepared for incubation: one for histidine, one for imidazole and one control population. Before incubation, the cells were counted at 5 million cells per ml.

For the probe incubation, a 25 mg/ml solution of imidazole in PBS was prepared, as well as a 10 mg/ml solution of histidine. These solutions were added to the cell culture flasks. For imidazole 0.8 ml probe solution was added to the cells along with 19.2 ml DMEM. For histidine 2 ml probe solution was added to 18 ml DMEM. This means that in both flasks, the probe concentration was 20mg per 20ml liquid, or 1mg/ml. To the control flask, 20ml of DMEM was added. The cells were incubated at 37°C for 24 hours. After this, the cells were washed with PBS, treated with trypsin to break the attachment to the flask, and then resuspended in PBS and transferred to NMR tubes.

Unfortunately, the peptide was not ready in time to include it in the incubations.

Results

4.1 Probe Synthesis

The next two sections deal with the acquisition of the deuterated probes. Imidazole and histidine were deuterated, and the peptide needed to be synthesised with the deuterated histidine.

4.1.1 Determining deuteration level

The level of deuteration in histidine and imidazole is expressed as a percentage of the total C2 and C5 atoms that are deuterated. Finding this percentage is trivial for histidine, but not so much for imidazole.

Histidine

To determine the deuteration in histidine, the side chain hydrogens, α and β in figures 4.1 and 4.2 can be used as a reference. These hydrogens are not deuterated with the process used. We can confirm this by looking at the ^{2}H spectrum of the deuterated compound (figure 6.3) where neither of these peaks are present. When the compound is deuterated, the hydrogens in the spectrum are replaced with deuterons, and will therefore no longer appear in the ^{1}H spectrum. The integral value of the two leftmost peaks should decrease, compared to the reference hydrogens. The amount by which they decrease gives the amount of deuteration that was achieved.

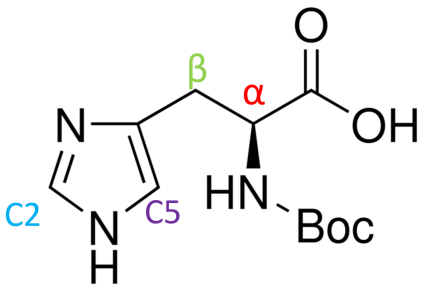

Figure 4.1: Boc-histidine with labels

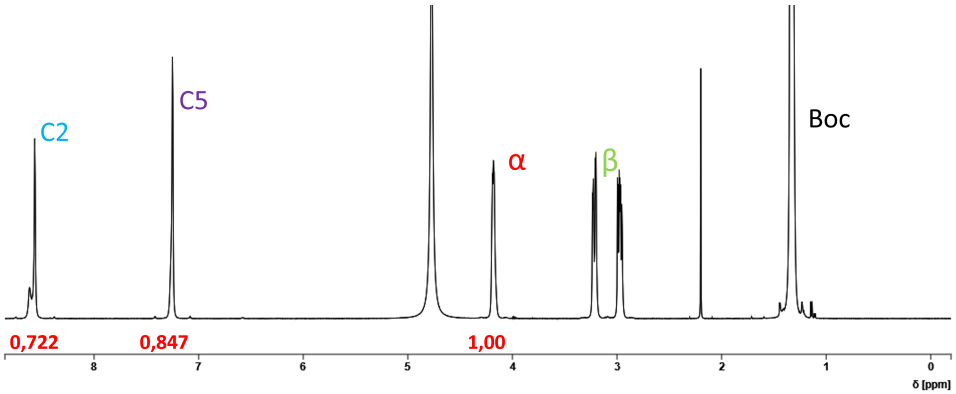

Figure 4.2: ^{1}H spectrum of undeuterated Boc-l-histidine

as an example, one of the early attempts at deuterating histidine using KOH is shown. The value of the C2 and C5 peaks has decreased dramatically to 0.089 and 0.109, respectively. This means that this deuteration has been very successful, with the deuteration on both the C2 and C5 positions 87%.

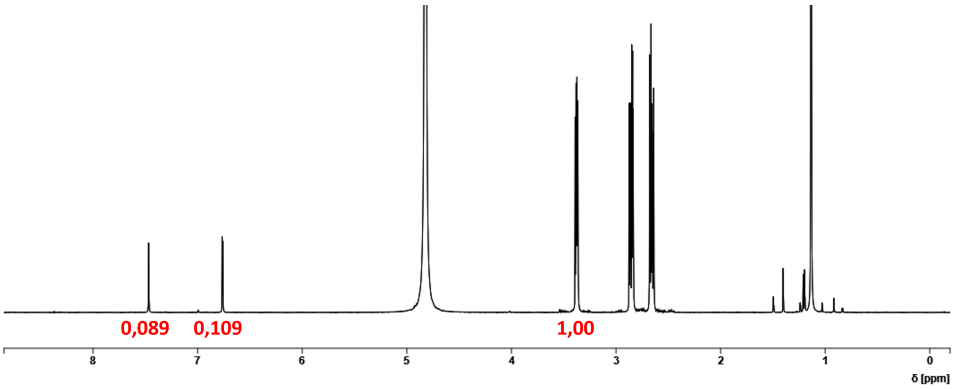

Figure 4.3: ^{1}H spectrum of KOH-deuterated Boc-l-histidine

However, this result is not usable since the KOH could not easily be separated from the product. Therefore the process was tried again with TEA as a base. The result is shown in figure 4.4. The C2 and C5 peaks have been reduced to 0.009 and 0.335. This corresponds to 99% deuteration on the C2 position and 60% on the C5. Although the result is not as good as in the KOH reaction, it is still adequate. A visible signal from the C2 position will more than likely mean there is also a visible signal from the C5 position, given that it is in the same order of magnitude. It is noteworthy that the integral values of the peaks do not match the ratio of 99% to 60% exactly, meaning that there is some unidentified inaccuracy.

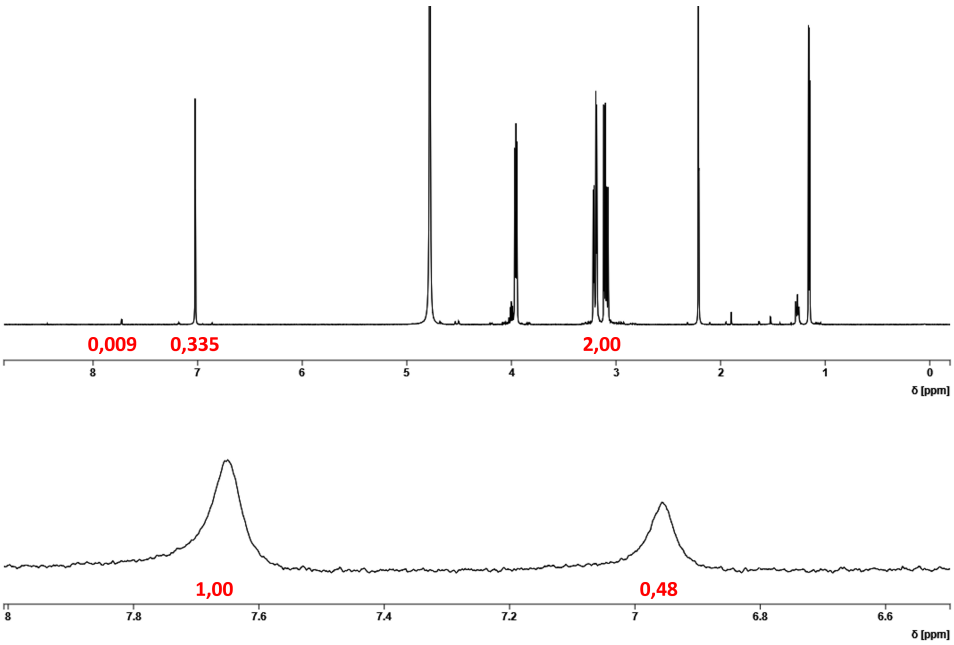

Figure 4.4: Above: ^{1}H spectrum of TEA-deuterated Boc-l-histidine
Below: ^{2}H spectrum of TEA-deuterated Boc-l-histidine

Imidazole

The ^{2}H spectrum of imidazole after deuteration is shown in figure 4.5

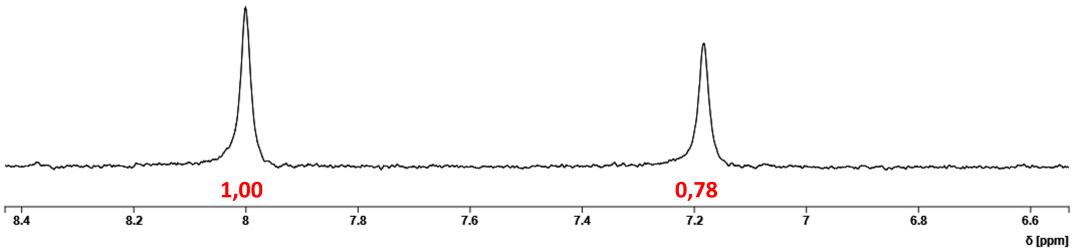

Figure 4.5: ^{2}H spectrum of deuterated imidazole

For imidazole, there is no simple quantitative method to determine the level of deuteration. However, we can estimate it based on a few things. We know from failed experiments, as well as the histidine deuteration, that the C5 is harder to deuterate than the C2 position. We can see this in appendix 1 figure 6.2 and 6.1 This tells us that the double deuteration that is visible likely means that the C2 deuteration is significant. Then, given the fact that the two peaks are in the same order of magnitude, we can deduce that the C5 deuteration is also significant.

4.2 Peptide synthesis

In order to determine if the peptide synthesis was successful, a mass spectrum was taken. (Figure 4.6)

The mass of the peptide should be 894u. The desired result would therefore be a peak at 894 in the spectrum. However, because potassium (mass 39) or sodium (mass 22) commonly affect the spectrum, a value of 933 or 916 is also a good result. As we can see though, these values do not appear in the mass spectrum. What we do see is a peak at 756. This seems to correspond to the hexapeptide created in the peptide synthesiser, which should have a mass of 754. This is within a possible range of inaccuracy. This means that the hexapeptide synthesis was successful, but not the manual addition step. Interestingly, there are also higher-numbered species present. This is unexpected since no products with a higher m/z than HAIYPRH should have been created.

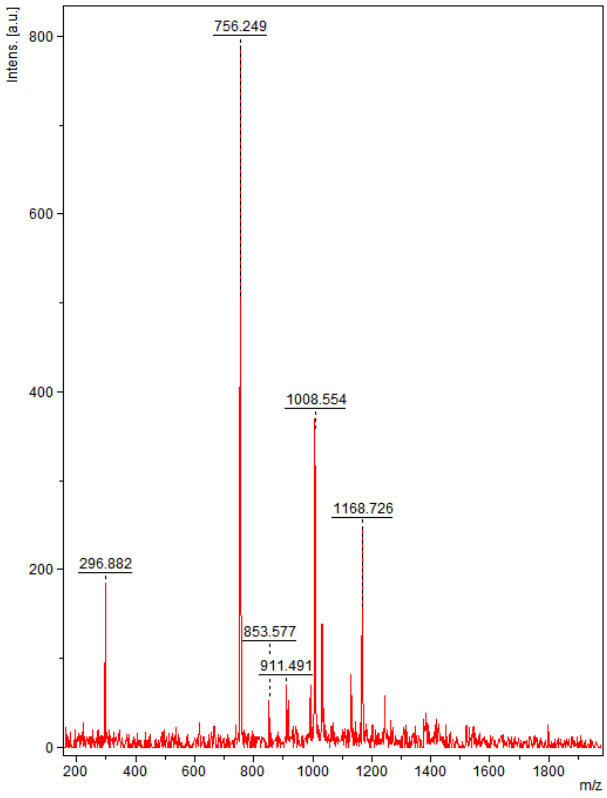

Figure 4.6: Mass spectrum of the peptide synthesis product

To determine what happened, a mass spectrum of deuterated histidine was taken. (Figure 4.7) The expected mass of deuterated histidine is 256, but in the spectrum, higher-numbered species are visible, meaning that unknown molecules are present. This could be explained by the fact that the histidine may have gotten too hot in the deuteration, as explained in the methods under section 3.1.2. The dry histidine was inadvertently heated to 110°C for at least 12 hours, which may have caused it to caramelise or burn. These processes could have created any number of byproducts, which may explain the unexpected molecules.

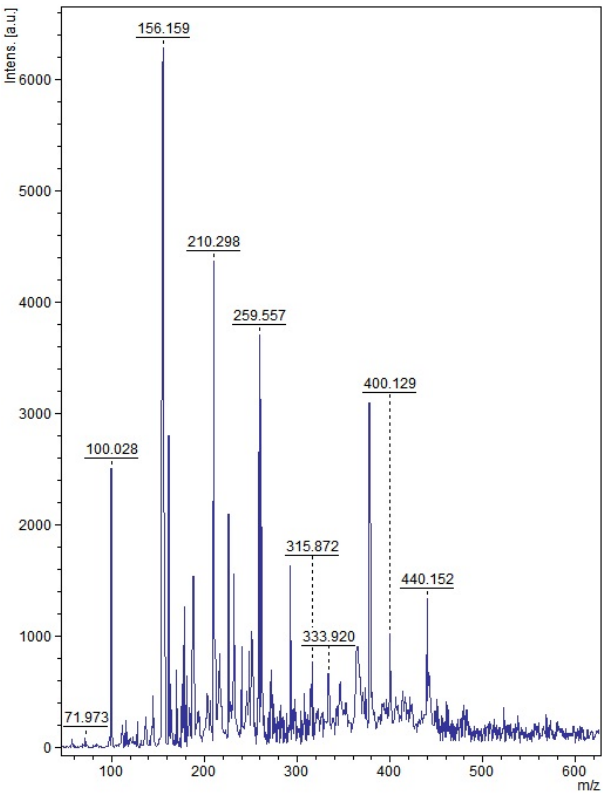

Figure 4.7: Mass spectrum of the deuterated histidine

4.3 Titration

In order to understand the pH behaviour of the probes in NMR, a titration was done.

4.3.1 imidazole

The titration of imidazole was done by obtraining a spectrum at 13 different pH values. The results are shown in figure 4.8, with the left peak representing the C2 deuterium atom and the right peak the C5 deuterium atom. The water peaks of these spectra have all been calibrated to 4.691 ppm, as this is the value of the water peak at a pH of 7.

What we can clearly observe is that as the pH of the solution changes, the NMR shifts of the deuterated positions change significantly in the physiological pH range of 6 to 8, and seem to follow a predictable pattern.

The crucial parameter is the distance in ppm between these two peaks, or $\Delta\delta$. It is plotted in figure 4.9 with a polynomial trendline drawn through the most linear region of the graph. This polynomial trendline has the formula

$$\begin{aligned} fit = {} & 0.0001583562703837x^6 - 0.0075619130127694x^5 + \\ & 0.1441809627399550x^4 - 1.3930725771770300x^3 + 7.1283985418962900x^2 \\ & - 18.2611534819413000x + 19.5100300797991000 \end{aligned} \quad (4.1)$$

which was generated using Microsoft Excel. $R^2 = 0.993$.

This graph shows that $\Delta\delta$ changes most in the region between 6 and 8. From this it can be concluded that deuterated imidazole is an ideal candidate for a pH probe because of its favourable pH characteristics: an observable change of its deuterium atoms in the physiological

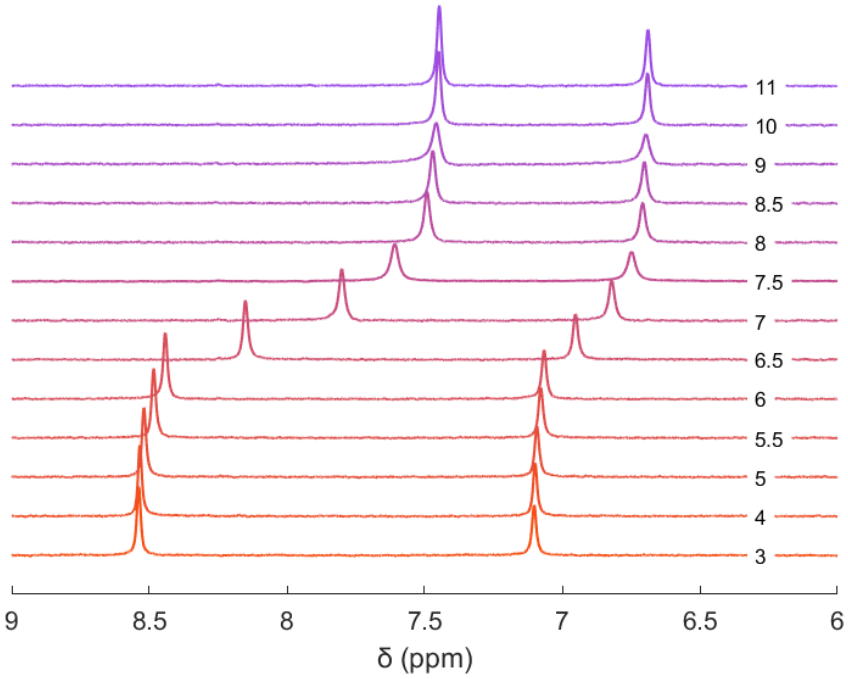

Figure 4.8: ^2H spectra in pH titration of imidazole

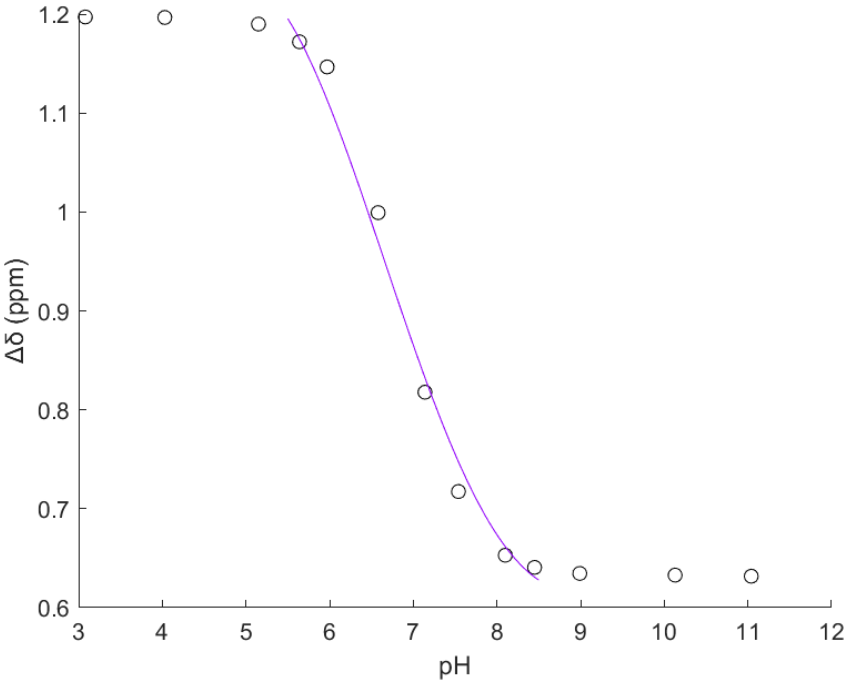

Figure 4.9: the pH dependence of $\Delta\delta$ in imidazole

pH range, which follows a predictable pattern. The trendline function will be used to determine the pH in the cell environment, as long as sufficient signal from its C2 and C5 deuterons is visible in ^{2}H NMR.

4.3.2 Histidine

For the titration of histidine, only 12 spectra were obtained, as the 7.5 pH spectrum was overlooked. The water peaks of the spectra were calibrated to 4.695 ppm. The results are shown in figure 4.10.

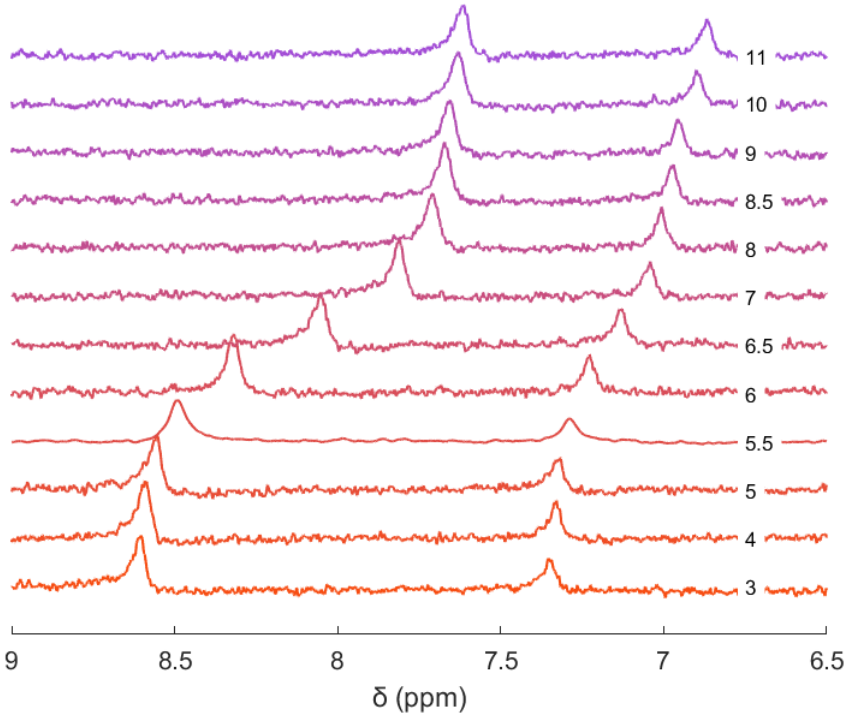

Figure 4.10: ^{2}H spectra in pH titration of deprotected histidine

A similar behaviour to that of imidazole is observed, except at the very ends of the pH range, where the peaks can be seen to get slightly closer together.

It is notable that the signal to noise ratio in these spectra is visibly worse than for imidazole. This can likely be explained by the overheating causing unwanted products to be formed in the histidine, lowering its solubility, aggravated by the fact that histidine has a higher molar mass and therefore a lower amount of C2 and C5 deuterons per milligram. This leads to a smaller amount of target deuterons being present in the NMR tube. An outlier in terms of noise is the pH 5.5 spectrum, on which some extra digital smoothing has been applied. It was deemed superfluous to repeat this process on all spectra as it does not improve the clarity of the results.

The change in distance between the peaks is plotted in figure 4.11. Once again a trendline has been added, with the formula

$$\begin{aligned} fit = {} & 0.000256445974287 * x^6 - 0.011204920979895 * x^5 + \\ & 0.195748022233468 * x^4 - 1.734779513392810 * x^3 + 8.151173449968330 * x^2 - \\ & 19.209220309490600 * x + 19.049751572833100 \end{aligned} \quad (4.2)$$

For this formula, $R^2 = 0.996$.

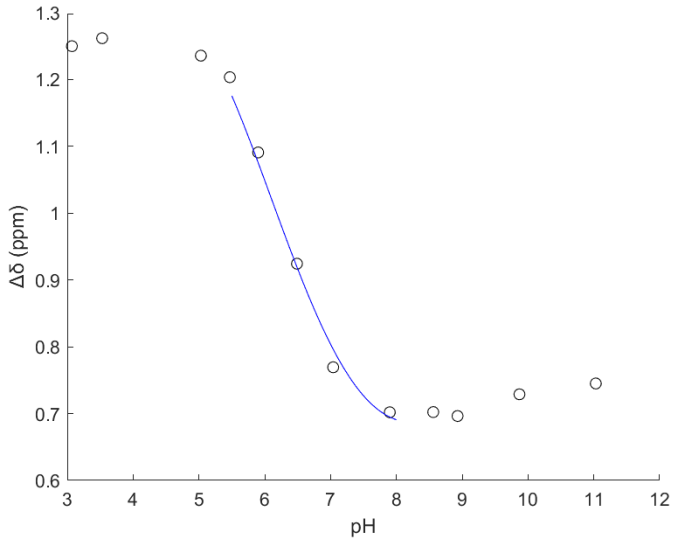

Figure 4.11: the pH dependence of $\Delta\delta$ in histidine

From these figures, we can conclude that histidine is also a suitable pH probe, granted that it is detectable in NMR.

4.4 Cell incubations

After 24 hours, the cell populations were examined. Microscope images are shown in figure 4.12. Although the focus is lacking, the confluence can still be estimated, at around 70% for the control, 75% for the imidazole-treated cells, and 30% for histidine. The poor viablilty of the latter may be explained by some residual THF present in the probe solution.

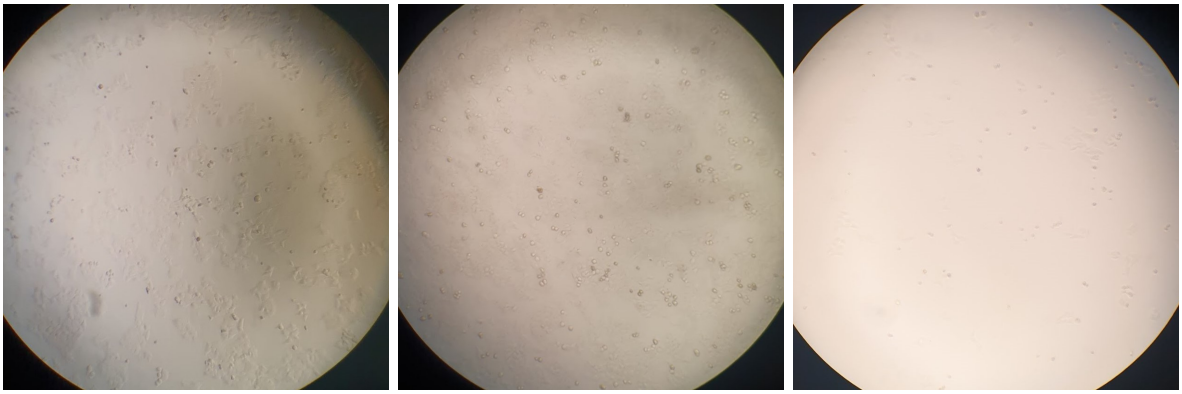

Figure 4.12: Probe-treated HeLa cells. L: Control, M: Imidazole, R: Histidine

Unfortunately, ^{2}H NMR spectra of the imidazole and histidine-treated populations showed no discernible presence of probe molecules. A 2-day acquisition was done, with the number of scans done by the machine totalling 32768, but even this gave no visible peaks between 7 and 9 ppm. This means that there was no cell uptake of the probes. A pH measurement could therefore not be attempted.

Conclusion

5.1 Discussion

The deuterations of histidine and imidazole have worked, but the cell toxicity of imidazole and the mass spectrum anomalies of histidine showed that more care should have been taken to obtain a clean product. The result of the cell experiments are somewhat invalidated by the fact that the probes were not as pure as was needed. The likely overheating of the histidine may have had an impact on its solubility, which complicated the titration, cell incubations, and may also have led to the peptide synthesis failing.

Another point of contention in this work is the lack of a clear direction in terms of intracellular or intercellular measurement. From the theory, it is clear that intercellular pH is the parameter of interest, as this is what is meant by the *tumour microenvironment*. However, as this project is a first proof of concept, either intercellular or extracellular measurements would have been a valuable result, as long as it is clear which was done. However, in a future experiment there should be more distinction between the two.

Lastly, the titrations showed very promising properties of imidazole and histidine, but in order to make accurate pH measurements, more data points should be taken. This will increase the accuracy of the curve fitting equations.

5.2 Conclusion

Unfortunately, the research question, *can deuterium NMR be used as a suitable method for determining intracellular pH in tumour cells*, can not yet be answered on the basis of this work. However, we do know that the technique works for ^{1}H NMR, and this research has given no indication that it will not work for deuterium NMR. Additionally, the potential of imidazole and histidine as deuterium NMR pH probes has been underlined. Titrations indicate that the pH characteristics of these two probes are very suitable for NMR pH measurement in the physiological range. Furthermore, the research done into deuteration methods of these probes has yielded promising results, as well as valuable lessons as to what strategies to avoid.

Overall, this research has yielded valuable new insights into the behaviour of pH probes in deuterium NMR, and a pathway into the clinical setting is imaginable.

5.3 Outlook

This research has exposed a number of new areas of research to explore. The experiment should be repeated with a number of changes. First of all, more care should be taken to avoid overheating of histidine, and to maintain the purity of imidazole. Furthermore, data is needed on the uptake and toxicity of imidazole and histidine to both cancer cells and healthy cells. Especially the maximum concentration of probe in the cell medium is important, as a higher concentration of probe on the cells may lead to better uptake and detection in the NMR. Also, data on healthy cell uptake is an important first step towards adapting this measurement technique to *in vivo* MRI experiments and eventually a clinical setting. More incubations should therefore be done, at varying probe concentrations and on more cell types.

Lastly, the peptide synthesis needs to be re-explored. The potential of creating a peptide with tailored properties for e.g. cell uptake adds a new dimension of possibilities and could be a great asset. For example, if an experiment is done that focuses clearly on intercellular measurement, a peptide could be very helpful in making sure that the probe does not enter the tumour's cells, but does stay firmly in its microenvironment.

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Appendix

failed deuteration without catalyst

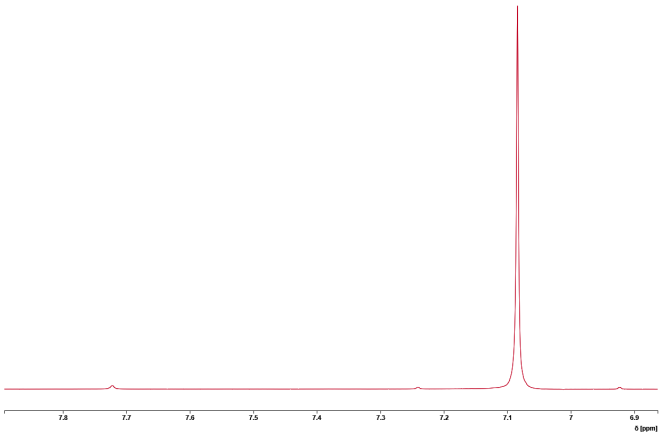

Figure 6.1: ^{1}H spectrum of attempted deuteration of imidazole without a catalyst

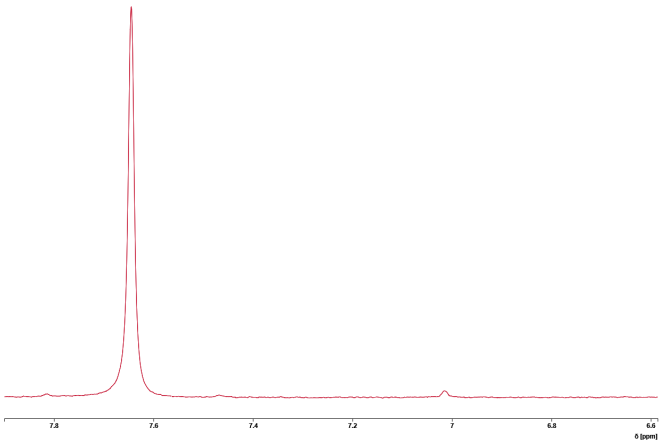

Figure 6.2: ^{2}H spectrum of attempted deuteration of imidazole without a catalyst

From the above spectra, we can tell that the C2 position (left) is nearly fully deuterated, since it almost doesn't show up in the 1H NMR and is very present in the 2H NMR. The opposite is true for the C5 position. This indicates that there is very strong deuteration of only the C2 peak, or monodeuteration.

Histidine spectrum

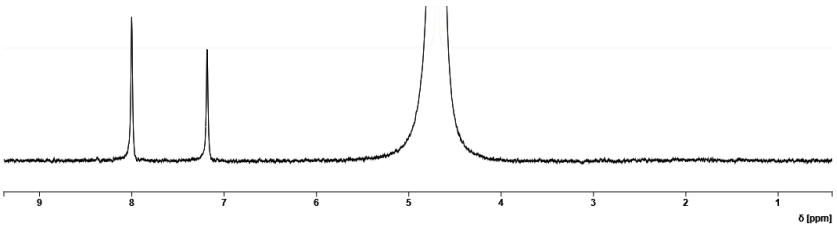

Figure 6.3: Full ^2H spectrum of TEA-deuterated histidine

Materials

Substance	**Manufacturer**
N_α-Boc-l-Histidine	Fluka
Deuterium oxide (D_2O), magnesium sulfate, Triethylamine (TEA)	Sigma-Aldrich
Trifluoroacetic acid (TFA)	Merck
Imidazole, potassium hydroxide (KOH), hydrogen chloride (HCl), dichloromethane (DCM)	unbranded

Table 6.1: Chemicals utilised and their origin

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