

Chapter 4

Experimental protocols for and studies of the effects of surface passivation and water isotopes on the gliding speed of microtubules propelled by kinesin-1

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Chapter 4

Water isotope effects on gliding motility assays

4.1 Introduction

Water is a crucial component to biomolecular interactions that is often overlooked [Parsegian 1995]. It can easily get through the lipid bilayer of cellular membranes. Cells are very densely packed and are regulated via protein generation and ion pumps so that they maintain a certain osmotic pressure. When cells are placed in a hypoosmotic environment, water will permeate the membrane such that the osmotic pressure inside the cell and outside it, balance. This can cause the cell to swell with water. The reverse case can occur as well when a cell is placed in a hyperosmotic environment. In this case, the water in the cell will leave in order to try and equilibrate the osmotic pressure differences. This causes the cell to shrink.

Water is needed for peptide hydrolysis and ATP hydrolysis. Kinesin uses water to hydrolyze ATP so that it can take steps along microtubules. Water is crucial for life and is necessary for many chemical reactions in cells. Water hydrates all protein membranes and has been found to be present when EcoRI non specifically binds to

DNA [Parsegian 1995]. When EcoRI does bind to the DNA, those water molecules that were in between the DNA and enzyme are expelled into the bulk water so that EcoRI can bind to the DNA. In order for the kinesin motor domain to bind to the microtubule during a step, it is reasonable to suggest that if it is hydrated, those water molecules would have to move from the protein surface to become bulk water molecules.

The work described in this chapter investigated if osmotic pressure changes can be observed using different isotopes of water while investigating the kinesin and microtubule system.

4.2 Methods and materials

Most of this section is identical to previous chapters. I will only describe the differences done with this assay in this section while linking to the relevant components from other chapters below.

- [Microscope & Image acquisition software](#)
- [Temperature stabilization](#)
- [PEM buffer](#)
- [Antifade & PEM-Glu](#)
- [Tubulin for Microtubule polymerization](#) & [Taxol](#) for microtubule stabilization.
- [Kinesin & PEM-A](#)
- [\$\alpha\$ -PEM](#).
- [Flow cells](#)
- [New PEM buffers using different isotopes of water.](#)

The differences for these experiments as compared to others I’ve done are subtle, however, they make a big difference to the assay. The measurable differences in how the assay responds to different environments is not too surprising considering how sensitive it is to ionic strength and pH [Böhm 2000b].

4.2.1 New flow cells

The construction of the new flow cells was very similar to the old construction. The only difference was that I intended for the new flow cells to not be permanently sealed. This required finding a material that would “seal” the flow cell in order to prevent evaporation and at the same time allow me to observe it on the microscope, remove it from the microscope, and flow in new material to be observed again. Surprisingly enough, plain old kitchen plastic wrap (cellophane) worked the best at this task.

Step 1: I created a [template](#) for fast and reproducible flow cell creation. Using my template, I centered a slide ([VWR 48300-025](#)) in the vertical rectangle.

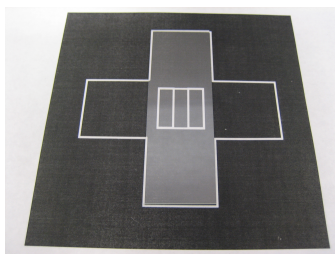


Figure 4.1: Step 1 in the flow cell construction.

Step 2: I added strips of permanent double stick tape ([Scotch](#)) to the slide by using the two most center lines as a guide. This created a channel that is approximately 10 μ L in volume. The width of the two center lines was 5 mm.

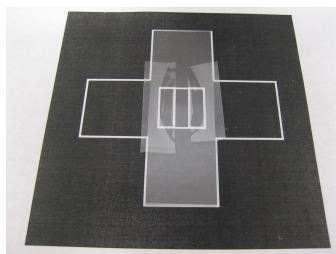


Figure 4.2: Step 2 in the flow cell construction.

Step 3: I trimmed the excess tape by using the outer lines of the center box as a guide. I did not trim the tape such that the only tape left on the slide was in the center box. I only trimmed the tape that hung over the slide. It turns out that keeping this extra tape is useful when making resealable flow cells. After trimming the tape I placed a cover slip ([VWR 48366-045](#)) over the center box. The center box was used as a guide to ensure that the cover slip was centered on the slide.

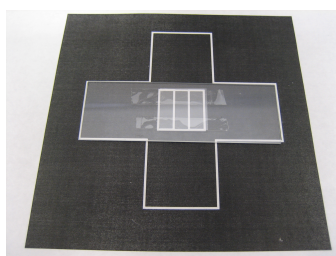


Figure 4.3: Step 3 in the flow cell construction.

Step 4: I ensured proper adhesion of the cover slip to the tape by pressing it to the slide. Proper adhesion was done when the light that is scattered from the slide + tape + slip combo differs from when the slip is merely placed on the tape. Compare the figure from step 3 to this step and notice the difference in how the light scatters from the center box.

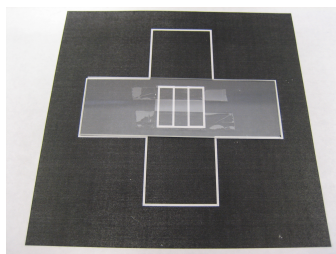


Figure 4.4: Step 4 in the flow cell construction.

Step 5: If the flow cell was not intended to be used as a resealable one, then it was finished Step 4. If it was intended to be used with the cellophane sealer, then I continued on with the following steps. Two very thin pieces of double stick tape were placed at the entrances of the flow cell. They have been colored red in the image to enhance contrast. These pieces of tape were essential for proper sealing with a piece of cellophane and helped prevent the objective oil from seeping into the flow cell. They can be made by holding two razor blades together and cutting a thin piece of tape. I had to ensure that the thin pieces of tape were as close as possible to the entrances of the flow cell since I observed excess evaporation occurred if they were not close.

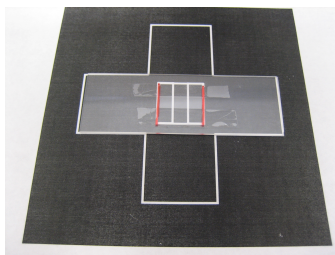


Figure 4.5: Step 5 in the flow cell construction.

Step 6: Small strips of cellophane ([Glad Cling Wrap](#)) were then placed over the flow cells and wrapped around the slide.

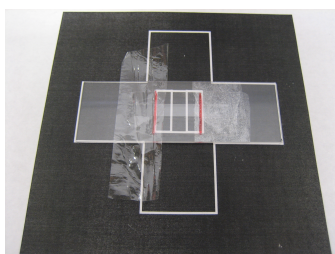


Figure 4.6: Step 6 in the flow cell construction.

I should note that I also tried static cling vinyl and pallet wrap before settling on using cellophane. The static cling vinyl was not used because it was too thick and did not properly seal the flow cell. But, it did indicate to me that the static cling was what I wanted to use as the new sealer for the flow cells. This lead me to pallet wrap

which was not used because it contains a small amount of glue and I did not want that glue seeping into my flow cells. Using it did show me that I needed a thinner material to “seal” the flow cell with and ultimately lead to the use of cellophane that can be purchased at a grocery store.

There has been some debate about the use of nail polish as the sealant for flow cells due to the organic solvents that are in them. Using both the nail polish sealant method and this new cellophane seal method (which has no chemicals that could possibly leach into the flow cell) did not show a difference in the observed gliding motility speeds. I will discuss this in greater detail and show a figure below in the Results section.

4.2.2 New solutions

Along with the 10x concentrated solution of PEM mixed in 18.2 M Ω -cm water, I prepared three other solutions containing two different isotopes of water, namely D₂O and H₂¹⁸O, and one sample with water that deuterium depleted and contained ≤ 1 ppm of D₂O. The reason for changing the water isotope was to try and measure speed differences dependent on the water isotope concentration in solution. The goal was to try and see if we could somehow relate the change in water isotopes to an increase in osmotic pressure of the system. I will discuss this more in the following sections however, the data is leading me to believe that I measured a viscosity change and not an osmotic pressure change.

D₂O overview

D₂O has some rather remarkable properties that are completely different than the properties of regular water. For instance, D₂O has the following properties ([[Hoefs 1987](#)], [[Soper 2008](#)], [[Katz 1957](#)]):

- 20 - 25% more viscous than H₂O.

- 10% more dense than H_2O .
- Boils at 101.42°C .
- Freezes at 3.81°C .
- Intermolecular bond longer than H_2O .
- Number of bonds in $\text{D}_2\text{O} \approx 3.76$.
- Number of bonds in $\text{H}_2\text{O} \approx 3.62$.
- $\approx \$1/\text{gram}$ at 99.9% purity.

The density of D_2O compared to the density of H_2O is easily visualized by how frozen D_2O will sink in a solution of H_2O . D_2O has also been shown to stabilize tubulin ([Chakrabarti 1999], [Das 2008]) and is known to prevent microtubules from depolymerizing [Panda 2000]. It is used in the thermal stabilization of vaccines [Sen 2009] and can even be used to make fruit flies more tolerant to heat [Pittendrigh 1974]. Rhodamine has been shown to have better photostability in heavy hydrogen water as well [Sinha 2002]. It is also known to pre-

vent cellular growth without changing the permeability of cellular walls to water ([Lucké 1935], [Lewis 1933], [Lewis 1934]). Deuterium is such an odd isotopic species that there have even been studies showing that a lack of deuterium during growth

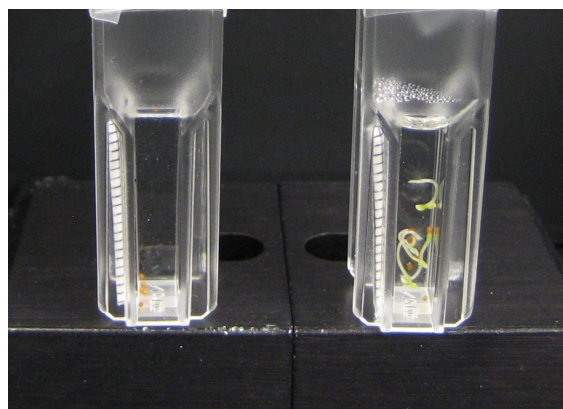


Figure 4.7: Recreation of the work done by Lewis in the 1930s. The cuvette on the left has tobacco seeds that have been soaking in 100% D_2O for 19 days and the one on the right has tobacco seeds that have been soaking in deionized water for 19 days. There is no apparent growth of the seeds in the left cuvette while there is definite growth in the seeds on the right.

causes stunting [Somlyai 1993]. In order to investigate the effects of D₂O on growth, I have started a side project that is attempting to recreate the seminal work done by Lewis ([Lewis 1933], [Lewis 1934]). Preliminary data shows that growth is affected by D₂O as can be seen in Figure 4.7.

Some work has been done on investigating how molecular motors act in a solution of D₂O [Chaen 2001]. Chaen et al. also showed that when they reduced the amount of ATP in solution such that it became the rate limiting step, the speed at which actomyosin converted ATP was less than the speed in H₂O.

H-PEM recipe

No studies have been made on how water isotopes affect kinesin and microtubules. To remedy this, I took data looking at the speed at which microtubules glide at in the presence of D₂O. I prepared a solution of PEM in D₂O which I called H-PEM to indicate that it contained the heavy hydrogen isotope of water. H-PEM was made in a 10x concentrated solution and contained the following.

- 800 mM of PIPES (6.0474 g PIPES)
- 10 mM of EGTA (.0951 g EGTA)
- 10 mM of MgCl₂ (250 μ L MgCl₂)
- \approx 1.25 M NaOH
- D₂O

I used NaOH to pH the solution but, pH is a concentration measurement of hydrogen ions in solution and not deuterium ions. In order to measure the correct “pD” of this solution using the pH meter, I had to add 0.41 to the measured value [Covington 1968]. This meant that the solution of PEM in D₂O was pH-ed to 7.30

using NaOH. H-PEM was passed through a 0.2 μm syringe filter, aliquoted, and then stored at 4°C in screw top vials.

H₂¹⁸O overview

Heavy oxygen water has the following properties ([Hoefs 1987], [Soper 2008], [Kudish 1972]).

- 5% more viscous than H₂O.
- 11% more dense than H₂O.
- Boils at 100.14°C.
- Freezes at 0.28°C.
- \approx \$200/gram at 97% purity.

There is not much in the literature about heavy oxygen water other than it is used extensively in labeling ([Schwartz 2007], [Dawis 1989]). Its diffusion through regular H₂¹⁶O water has been measured as well [Easteal 1984]. I believe that the lack of research using H₂¹⁸O is probably due to its high cost. A single gram of water with 97% atomic purity of ¹⁸O costs \$200. A gram with 99% atomic purity of ¹⁸O costs nearly \$1000 at the time of this writing. With this type of cost prohibitions, I had to change the way I prepared PEM in H₂¹⁸O.

O-PEM recipe

To prepare the PEM solution using heavy oxygen water I actually diluted the 10x PEM into the H₂¹⁸O water. This allowed me to make a solution of PEM containing 90% H₂¹⁸O. To make this solution I did the following.

1. 100 μL of 10x PEM
2. 900 μL of H₂¹⁸O

I should note that the vial in which H_2^{18}O comes in makes it extremely difficult to remove all the material. Because of this, I was only able on average to acquire 850 μL of H_2^{18}O water from a single vial. Using the techniques outlined in the previous chapters, I was able to run 30 different assays using varying amounts of H_2^{18}O water in the assay with only 2 stock vials of heavy oxygen water. This solution was not pH-ed and it was not filtered for fear of losing too much material in the filter. It aliquoted it and stored it at 3°C .

D-PEM recipe

Our reasoning behind investigating this one data point was two fold. We wanted to see what would happen if there was an almost zero amount of D_2O in solution and to use the deuterium depleted water for our tobacco seed experiment. In order to maintain the absolute minimum amount of D_2O in any solution, all solutions were remade with the deuterium depleted water. This meant remaking the following solutions in deuterium depleted water ($\text{D-H}_2\text{O}$).

- 10x PEM in $\text{D-H}_2\text{O}$ (10x D-PEM) — This meant preparing a 1 M solution of MgCl_2 in $\text{D-H}_2\text{O}$.
- 1.0 mg/mL α -casein in D-PEM (α -DPEM)
- 0.5 mg/mL α -casein in D-PEM (DPEM- α)
- 100 mM ATP in D-PEM (DPEM-A)
- 2 M glucose in D-PEM (DPEM-Glu)
- 1 mM ATP in DPEM- α (DPEM- α A)
- 20 mg/mL glucose oxidase in D-PEM (DPEM-GOD)
- 8 mg/mL catalase in D-PEM (DPEM-CAT)

- Antifade using DPEM-GOD and DPEM-CAT

Preparation of these samples was conducted in the same manner outlined in [Chapter 2](#). The only differences with what was mentioned in [Chapter 2](#) and this assay was that I had to mix up a 1 M solution of MgCl_2 . Adding MgCl_2 to water produces a highly exothermic reaction and much to my surprise, I found out how exothermic this reaction was.

The only possible sources of D_2O came from the kinesin, which the stock solution was not stored in deuterium depleted water, and/or from the microtubules since the tubulin was stored in [TSB](#) which did not use deuterium depleted water.

4.3 Experiment and data analysis

The experimental procedure and data analysis of this experiment is nearly identical to the steps taken in [Chapter 3](#). Below, I will outline the experimental procedure since it does differ from what was done there and is relevant to the discussion. However, I will only link to the data analysis section of [Chapter 3](#) below.

4.3.1 Experiment

Experimental flow cells for observation were prepared as follows.

1. Prepared a flow cell. Either the type to be sealed with nail polish or the type to be sealed with cellophane.
2. Incubated the flow cell with 1.0 mg/mL alpha casein in PEM for 10 minutes.
3. During the 10 minute incubation, [kinesin](#) was diluted in 0.5 mg/mL [α-casein](#) in PEM plus 1 mM ATP. The final concentration of kinesin used in these experiments was 27.5 $\mu\text{g/mL}$.

4. After 10 minutes, kinesin was flowed into the flow cell by fluid exchange. The volume of the flow cell was approximately 10 μL . To ensure that all the fluid in the flow cell was exchanged, 20 μL of the kinesin mixture was flowed into the chamber. This was then allowed to sit for 5 minutes.
5. During the various incubation steps, a new motility solution was prepared for each assay. The motility assays consisted of:
 - 1 μL of [PEM-A](#).
 - 1 μL of [PEM-Glu](#).
 - 2.5 μL of [Antifade](#).
 - 0.1 μL of [Taxol](#).
 - Some percentage of either O-PEM or H-PEM.
 - The necessary volume of [PEM](#) required to make the motility solution a final volume of 100.1 μL .
 - 5 μL of [microtubules](#).
6. After the 5 minute incubation with kinesin, 20 μL of the motility solution was introduced to the flow cell by fluid exchange.
7. The flow cell was either sealed with nail polish or cellophane dependent on which assay was to be performed.

After sealing the flow cell, the slide was immediately placed on the microscope for observation. For a detailed description of why I used these parameters, please see [Chapter 3](#). Briefly, the camera was set to take images as follows.

- 600 total frames which was approximately 2 minutes total exposure for a single ROI.
- EMCCD gain of 150.

- 100 ms single exposure.
- Frames were taken at 5 frames/second.

D-PEM assay

The same steps to produce the D-PEM assays were taken as was outlined above. I will list how the D-PEM assay was performed such that possible sources of D₂O can be highlighted.

1. A flow cell was made and incubated with α -DPEM for 10 minutes.
2. During the casein incubation, a dilution of kinesin in DPEM- α A was made. I added 2 μ L of the kinesin stock to the DPEM- α A solution. The final concentration of kinesin was again 27.5 μ g/mL, however, this step gives a possible source of D₂O.
3. After 10 minutes, 20 μ L of DPEM- α A + kinesin was flown into the flow cell and allowed to incubate for 5 minutes.
4. During the incubation periods, a new motility solution was prepared containing the following.
 - 90.5 μ L of D-PEM
 - 1 μ L of DPEM-A
 - 1 μ L of DPEM-Glu
 - 2.5 μ L of antifade made from the DPEM-GOD & DPEM-CAT stocks.
 - 0.1 μ L of Taxol
 - 5 μ L of polymerized microtubules that were fixed in a solution of 10 μ M Taxol in D-PEM.

5. After the 5 minute incubation, 20 μL of the motility solution was added to the flow cell.
6. The flow cell was sealed with nail polish and immediately observed on the microscope.

The sources of D_2O from this assay come from the kinesin and motility solution steps. Presumably, the fluid exchange from the kinesin to the motility solutions removes all the fluid from the flow cell. If it does, then the only source of D_2O in the assay would come from the TSB. The tubulin stored and polymerized in TSB is diluted by 200% when fixed with Taxol after polymerization. Those microtubules are again diluted into the motility solution by 95%. This leaves an approximate percentage of 0.5% of possible D_2O contamination, or 0.04 mM of D_2O in solution. The maximum possible contaminant concentration of D_2O in this solution is 20% or 1.6 mM.

4.3.2 Data analysis

Please see [Chapter 3](#)'s data analysis section for a detailed description of how the data was analyzed.

4.4 Results and discussion

4.4.1 Results

Each slide was observed for a total of 15 fields of view, or about 30 minutes for each slide. Assays were run at 10% increments of H-PEM added to the motility solution starting from 0% and ending with 90%. Each 10% increment was then run a total of 3 times. For each slide, only the last 10 fields of view were kept to compute an average speed value for that assay. The first 5 data points were removed due to the increase in temperature of the slide from room temperature (24°C) to the objective

at 33.1°C. Averaging those 10 data points gave a single data point for the assay. Each assay was then run three separate times. This gave a total of 3 average velocity data points that were the accumulation of hundreds of tracked microtubules for a single assay.

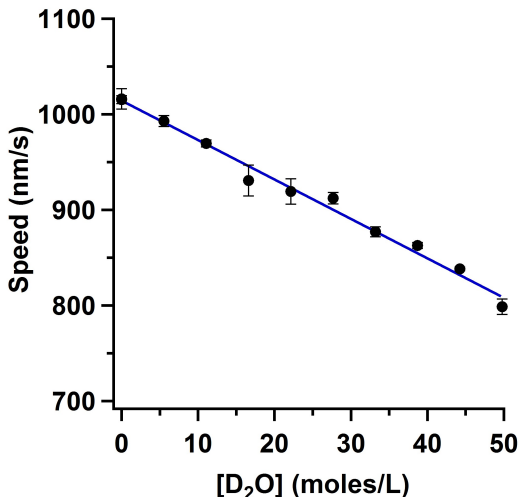


Figure 4.8: Graph showing the gliding speed dependence of microtubules when D₂O was added to the motility solution.

Each data point in Figure 4.8 is the average of those 3 speed measurements and the error bars are the standard error of the mean (SEM). The concentration of D₂O was calculated by first converting the percentage values of H-PEM used in the motility assay to concentration values in one mole of H₂O. Next, the baseline amount of D₂O in water was calculated to be 8 mM [Somlyai 1993]. The 8 mM baseline was then added to the concentration of H-PEM used.

The assay that used D-PEM as the buffer gave a speed value that was indistinguishable from the “zero” value that had 0% H-PEM as the solution, 1015 nm/s. This result was as expected noting how linear Figure 4.8 is.

Exchanging the D₂O for H₂¹⁸O in the motility solutions also showed a remarkably linear graph, Figure 4.9. The concentration of H₂¹⁸O was calculated in a similar fashion as was the D₂O concentration.

Data taken with the resealable flow cells that used cellophane as the “sealant”

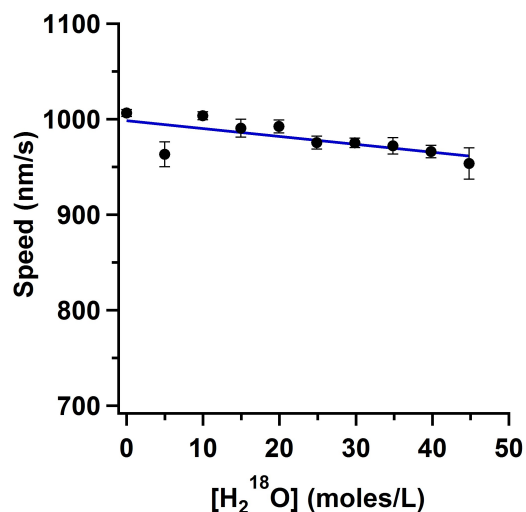


Figure 4.9: Graph showing the gliding speed dependence of microtubules when H_2^{18}O was added to the motility solution.

is shown in Figure 4.10. The small grey markers are the speed measurements for the individual assays that were conducted. The blue lines are the average speed measurements determined from those assays using the same methodology described above. The first cluster of data are speed measurements using no H-PEM in solution. The second, is when the 0% H-PEM solution in the flow cell was exchanged with 90% H-PEM. The third cluster is when the 90% H-PEM was exchanged for 0% H-PEM.

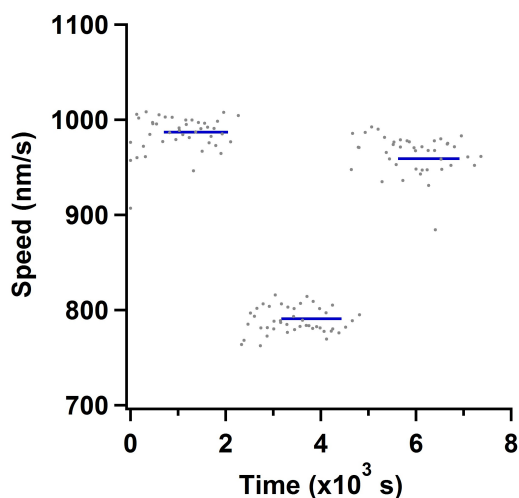


Figure 4.10: Graph showing the gliding speed dependence of microtubules using the resealable flow cell. Data was taken using 0% concentration of H-PEM then 90% concentration of H-PEM and back to a 0% concentration.

4.4.2 Discussion

The linearity of the assays run with D_2O and $H_2^{18}O$ are quite remarkable. The decrease in speed for the D_2O assay is about 21% and the decrease in the $H_2^{18}O$ assay is about 5%. The viscosity of D_2O is about 20% greater than H_2O while $H_2^{18}O$ is 5% greater than H_2O . The speed differences measured would suggest (in light of the viscosity numbers) that we measured a viscous change in the solvent and not an osmotic pressure change. If viscosity is the cause of the decrease in speed, then these results are quite remarkable as they indicate that gliding motility speeds can measure small viscous changes in solutions. More investigation needs to be done in order to fully understand what is happening because the results may not be due to a viscous effect.

Kotyk et al. [Kotyk 1990] showed that ATP activity was not affected by the addition of D_2O using yeast as the test subject. Our data would suggest that ATP activity of kinesin is also not affected by the addition of D_2O . If ATP activity was hindered by the addition of D_2O then I would have expected to see slower speeds than what I measured using D_2O . I did not make any measurements using less than the 1 mM ATP in the motility solutions so ATP hydrolysis was never the rate limiting step.

The use of nail polish as the flow cell sealant has been debated in our lab before. We use it because it is convenient and it works well. I have seen and heard of others using hot wax as the sealant and or even using glycerol. The data where I used cellophane as the flow cell sealant, shown in Figure 4.10, show that using nail polish as the sealant is completely acceptable since the first cluster of data points gave speed measurements that were similar to those done with nail polish.

Figure 4.10 also shows that speed measurements using 90% H-PEM do not damage the system. The speed difference between the two 0% H-PEM washings is odd, however, and can probably be explained by not removing all the D_2O in the flow

cell. I washed the flow cell with only 2 sample volumes and there may be a change in measured speeds if more sample volumes were used in the washing.

4.5 Conclusion

The goal of this experiment was to investigate the kinesin and microtubule system using different isotopes of water. Our initial thought was that by changing the water, we would change the osmotic pressure of the system. The data show that by changing the isotope of water used in the motility assay caused the gliding speeds of microtubules to change in a manner that we have not been able to determine a suitable description for.

I also noted that using nail polish as the sealant for flow cells does not change the speed at which microtubules glide at. Nor does using D₂O damage the system.

4.6 Future work

In order to measure osmotic pressure changes I can use two different osmolytes in the gliding motility assay that have very different viscosities. For example, glucose has a high viscosity as compared to betaine. Betaine is an osmolyte that your cells use to change their osmotic pressures. In order for betaine to have the same viscosity as a solution of glucose, I would have to use a lot more betaine than glucose. The concentration difference of glucose and betaine in the gliding motility assay would mean that the two solutions would have different osmotic pressures. It will be interesting to see if I can measure speed differences using two solutions with different osmolyte concentrations that have the same viscosity.

Finally, in order to ascertain if the speed differences measured using the resealable flow cell after washing with H-PEM is due to D₂O contamination, I could redo the experiment except with a lot more fluid exchange than just two sample volumes.

References

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