Effect of ^{2}H and ^{18}O water isotopes in kinesin-1 gliding assay

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Abstract

We show here the effects of heavy-hydrogen water (^2H_2O) and heavy-oxygen water ($H_2{}^{18}O$) on the gliding speed of microtubules on kinesin-1 coated surfaces. Increased fractions of isotopic waters used in the motility solution decreased the gliding speed of microtubules by a maximum of 21% for heavy-hydrogen and 5% for heavy-oxygen water. We discuss possible interpretations of these results and the importance for future studies of water effects on kinesin and microtubules. We also discuss the implication for biomolecular devices incorporating molecular motors.

Introduction

Water plays a crucial role in the interactions of biomolecules, especially when biological surfaces bind and unbind. When binding surfaces are apart, the neighboring water molecules form a hydration shell around the surfaces that is more ordered and less dynamic than the bulk water molecules [1]. Changes in osmotic stress affect the rate of transport of water between the hydration shells and the bulk water [2]. Thus, on- and off-rates for surface-surface binding is strongly affected by changes in the activity of bulk water. As noted by Parsegian *et al*., water activity changes that affect on- and off-rates are often overlooked, even in meticulous biophysical studies [3]. In fact, water is an often-overlooked yet vitally important aspect for biophysical systems and is "the most important problem in science that hardly anyone wants to see solved" [4]. A good example of the profound importance of water activity is shown by a more than three orders of magnitude increase in binding lifetime in a protein-DNA complex when the osmotic stress is increased by a few molar betaine [5]. The effects of water are even less studied in the molecular motors field, and in particular, for the kinesin-1 and microtubule system.

Though understudied for molecular motors in general, there have been some experiments indicating the importance of water effects on actin and myosin. For example, Highsmith *et al*. performed a detailed study on the effects of osmotic stress on the activity of myosin and binding to actin [6]. They demonstrated that changing osmotic pressure was effective for probing water molecules at the binding interface. The authors also point out the importance of the increased osmotic pressure inside living cells. Chaen *et al*. also showed a 60% reduction in *in vitro* actin gliding velocity in heavy-hydrogen water [7-8].

We are unaware of prior studies involving either use of water isotopes, or investigation of osmotic stress effects using the kinesin and microtubule system. However, there have been several studies of these effects on tubulin alone and microtubule polymerization. It is well-known that osmotic stress promotes microtubule polymerization and a high percentage of glycerol is often used to promote in vitro microtubule polymerization [9]. It has also been shown that heavy-hydrogen water promotes tubulin polymerization and stabilization [10-11]. Heavy-hydrogen water stabilizes polymerized microtubules, which has been shown to be a likely reason for why deuterium oxide is highly toxic to eukaryotic organisms [12]. Lewis initially demonstrated the toxicity of deuterium oxide in the 1930's [13-14]. The ability of tubulin to polymerize fades rapidly when stored in normal aqueous buffer at 4ºC. Heavy-hydrogen water dramatically reduces this instability allowing tubulin to polymerize even after storage at 4ºC for 2 days [15]. Heavy-hydrogen water has also been shown to stabilize a wide range of biomolecules and has potential technological implications for vaccine stability [16]. It is likely that heavy-hydrogen water has a general stabilizing effect on biomolecules and biomolecular complexes as it has also been shown to protect fruit flies from elevated temperature [17].

Despite its importance, it can be argued that water solvent effects have seen relatively little attention in the kinesin and microtubule system. Water isotopes have been used, but usually as spins or tracers rather than a probe of water activity [18]. In this paper, we show that heavy-hydrogen water and heavy-oxygen water each slow down microtubule gliding speeds on kinesin-1 surfaces. Thus, water isotopes may provide an important experimental knob to turn while studying the effects of water interactions on the behavior of kinesin-1 in cells and biomolecular devices.

Methods and Materials

[Note to Editor and Referees: We are currently working with Figshare.com to post the entire data sets contained in this paper, from the raw images to the final processed figures. This is about 300 GB of image series, movies, tracking data, etc. We are currently out of room on our public server to display this data, but have not yet succeeded in uploading to Figshare.com. We anticipate that this can be accomplished during review. If referees / editors would like to see similar data sets, they can be found in data supporting our previous PLoS ONE publication, data available at http://kochlab.org/files/passivation/ Maloney A, Herskowitz LJ, Koch SJ (2011) Effects of Surface Passivation on Gliding Motility Assays. PLoS ONE 6(6): e19522. doi:10.1371/journal.pone.0019522]

Microscope

Experiments were conducted on an Olympus IX71 inverted microscope using an Olympus 60x 1.42NA PlanApo objective. Rhodamine fluorophores attached to tubulin were illuminated using a 100W mercury lamp and excited using a TRITC filter cube from Chroma (Chroma 49005). The light illuminating the fluorophores was attenuated by 94% in order to prevent excessive photobleaching. The objective was temperature stabilized to achieve consistent microtubule speed measurements. A custom objective heater was constructed for the temperature stabilization and for a more in depth description of the heater please see Maloney *et al*. [19]. Briefly, the objective heater used a polyimide film resistive heater to heat the objective using control circuitry from TeTech (TC-48-20). LabVIEW software supplied by TeTech was used and modified to suit our time-stamping requirements. Temperature stabilization plays a crucial role in obtaining stable measurements [18, 20-22], however, many studies do not indicate the temperature at which speed measurements were observed. All our experiments were conducted at 33.1 ± 0.1ºC. Image sequences were captured every 200ms for a total of 600 frames with an Andor Luca S EMCCD camera. Images were stored in “png” format using custom LabVIEW software.

Flow cells

Flow cells were constructed using two strips of double stick tape (Scotch), sandwiched between a microscope slide (VWR 48300-025) and a cover slip (VWR 48366-045). The channel formed between the pieces of tape was approximately 10μL in volume and was sealed using nail polish, which prevented excessive evaporation of the motility solution. In order to determine if heavy-hydrogen water affected speed measurements, we also created flow cells that could be “sealed” and “unsealed” with cellophane. A complete description of our procedure can be found in Text S1. Briefly, resealable flow cells were constructed in the same manner as sealable ones with the addition of two very thin pieces of double stick tape placed on top of the slip and at the entrances of the channel. Cellophane (Glad Cling Wrap) was then wrapped around the entrances of the flow cell in order to prevent the motility solution from evaporation.

Buffers and solutions

We used four different solvents to make the buffer solutions used in our experiments; light water (H_2O), heavy-hydrogen water (^2H_2O), heavy-oxygen water ($H_2{}^{18}O$), and deuterium-depleted light water.

The light water buffer was made at a 10x concentrated solution and contained: 800mM PIPES (Sigma 80635), 10mM EGTA (Sigma 80635), 10mM $MgCl_2$ (Sigma M1028) and pH-ed to 6.89 using approximately 1.25M NaOH (Fisher S318). The light water used for this buffer had a resistivity of 18.2MΩ-cm and was produced with a Barnstead EasyPure RoDI system. The light water buffer was passed through a 0.2µm syringe filter and aliquoted in 2mL screw-top vials, stored at 4ºC, and stored for no longer than 6 months.

Another 10x concentrated buffer solution containing the same 800mM PIPES, 10mM EGTA, and 10mM $MgCl_2$ was prepared using heavy-hydrogen water (Sigma 151882) and pH-ed to a value of 7.30 using NaOH. In order to report the correct pD value of a solution containing heavy-hydrogen water, 0.41 must be added to the measured value of pH [23]. Thus our solution had a pD value of 7.71, which is higher than the pH value for the light water buffer. The difference in the pH values of these two buffers and the consequence of differences in the measured speed value is discussed below. The heavy-hydrogen buffer was syringe filtered using a 0.2µm filter and aliquoted in 2mL screw-top vials, stored at 4ºC, and stored for no longer than 6 months**.**

The third buffer solution contained heavy-oxygen water (Sigma 329878). A 90% dilution of the 10x light water buffer was added to the heavy-oxygen water in order to create a solution containing 80mM PIPES, 1mM EGTA, and 1mM $MgCl_2$, the motility buffer conditions. We diluted the light water buffer into the heavy-oxygen water due to the high cost of the heavy-oxygen water. The solution was not filtered for fear of losing too much material and stored at 4ºC for no longer than 6 months.

The fourth buffer again was a 10x concentrated solution and contained 800mM PIPES, 10mM EGTA, 10mM anhydrous $MgCl_2$ (Sigma 449172) and pH-ed to 6.89 with NaOH using deuterium-depleted light water as the solvent (Sigma 195294) which was quoted to have less than 1 ppm deuterium oxide in solution. The deuterium-depleted light water buffer was made with anhydrous $MgCl_2$ in order to reduce the amount of contaminant deuterium in solution. It was syringe filtered using a 0.2µm filter and aliquoted into 2mL screw-top vials, stored at 4ºC and stored for no longer than 6 months.

1x concentrated solutions of the light water buffer, heavy-hydrogen buffer, and deuterium-depleted buffer were made with the addition of 1mg/mL bovine α-casein (Sigma C6780) in solution. Bovine α-casein was chosen as the surface passivator as it has been shown to give consistent gliding motility assays [18], however, it is not the only passivator that can be used to conduct gliding motility assays [24]. No α-casein solution using the heavy-oxygen buffer was made due to the cost of the $H_2{}^{18}O$ water. All α-casein solutions were mixed for approximately one hour at room temperature using a stir plate until no visible precipitates of α-casein were left in solution. These solutions were not filtered and were aliquoted into 2mL screw-top vials and stored at 4ºC. No investigations were made to determine the solubility of α-casein in the various water buffer solutions.

Tubulin was polymerized into microtubules using a Thermo PCR Sprint thermal cycler held at a constant temperature of 37ºC for 30 minutes. The tubulin used for polymerization consisted of 29% rhodamine-labeled bovine tubulin (Cytoskeleton TL331M) and 71% unlabeled bovine tubulin (Cytoskeleton TL238) at a concentration of 5mg/mL. The polymerization solution was a total volume of 1µL and contained: light water buffer, an extra 1mM $MgCl_2$, 1mM GTP (Sigma G8877), 6% (v/v) glycerol (EMD GX0185) and tubulin. Adding an excess of 1mM $MgCl_2$ to the

light water buffer ensured the EGTA did not chelate all the magnesium ions from solution since tubulin polymerization requires magnesium ions [25]. Tubulin will polymerize in the presence of ATP; however, GTP is known to be a better nucleotide for polymerization [26]. Glycerol acts as an osmotic stress in the polymerization solution and helps to speed up the polymerization process [9]. Microtubules were formed after 30 minutes in the thermal cycler and diluted by 200x with a solution of 10μM Taxol™ (Cytoskeleton TXD01) in light water buffer. Taxol™ is a non-polar chemical that must first be dissolved in DMSO (Sigma D2650) before adding to the light water buffer. Taxol™ has been shown to create crystals in aqueous solutions due to its low solubility that appear to be fluorescent microtubules [27]. It also has a high affinity for free tubulin and/or rhodamine dye molecules [28-29]. In order to prevent Taxol™ crystals from forming in solution, all solutions requiring Taxol™ were prepared immediately before each experiment and no stock solutions containing Taxol™ in an aqueous environment were stored for more than the duration of daily experiments. Microtubules were left at room temperature for the duration of an experiment in order to prevent depolymerization [9].

Dr. Haiqing Liu generously supplied Kinesin to us in 20μL aliquots at a concentration of 0.275mg/mL kinesin. The kinesin was his-tagged, truncated kinesin-1 dmk401 [30-31]; from drosophila and was expressed in *E. coli*. Kinesin was diluted to 27.5μg/mL for each assay.

Motility assays

All motility assays contained the following components; 10μM Taxol™, 1mM Mg-ATP (Sigma A9187), 20mM D-glucose (Sigma 49139), 2.5% (v/v) of an oxygen scavenging antifade cocktail, and 5mL of fixed polymerized microtubules. The antifade cocktail was a dual enzymatic oxygen scavenging system, which consisted of; 800 mg/mL glucose oxidase (Sigma G6641), 2000 mg/mL catalase (Sigma C9322) and 20% (v/v) of 2-mercaptoethanol, BME, (Sigma 63689). When diluted into the motility solution, there was 8 mg/mL glucose oxidase, 20 mg/mL catalase and 0.5% (v/v) BME. Antifade cocktails were prepared in advance and stored in 5mL aliquots at -20ºC and used within one week.

Experiments using light water required the flow cell to be passivated with 1mg/mL α-casein in the 1x light water buffer for 10 minutes. Similarly, experiments using heavy-hydrogen water were incubated with 1mg/mL α-casein in the 1x heavy-hydrogen buffer, and those experiments using deuterium-depleted light water used 1mg/mL α-casein in the 1x deuterium-depleted light water buffer. 1mg/mL α-casein in the light water buffer was used as the passivator in the heavy-oxygen buffer experiments.

Each experiment incubated the flow cells at room temperature, 24ºC, for 10 minutes. During this 10-minute incubation, kinesin was diluted to 27.5μg/mL in a 20μL solution of either 1x light water buffer, 1x heavy-hydrogen buffer, heavy-oxygen buffer, or 1x deuterium-depleted light water with the addition of 1mM Mg-ATP and 0.5mg/mL α-casein. After the 10-minute incubation with α-casein, the 20μL kinesin solution was introduced to the flow cell by fluid exchange. The flow cell was then allowed to incubate for another 5 minutes. During this second incubation time, a motility solution was prepared using the different water isotope buffers. After the 5-minute incubation, 20μL of the motility solution was flown into the flow cell by fluid exchange. The flow cell was then sealed and observations were conducted immediately on the microscope. All observations were done in the center of the flow since this area gave the most consistent gliding motility speeds in our flow cells [18].

Experiment and data collection

Images were captured every 200ms. A total of 600 frames were captured for a given region of interest (ROI) using an EMCCD gain of 150 with an Andor Luca S camera. The illuminated ROI was observed for approximately 2 minutes. The use of the antifade, attenuation of the mercury lamp, and proper Köhler illumination helped prevent excessive photobleaching of the sample. A total of 15 ROIs were taken for each slide prepared. Due to the incubation of the flow cells at room temperature, it took approximately five 2 minute intervals until the slide reached a stable temperature and consistent speed measurements were achieved. Each water condition was repeated for a total of 3 independent flow cells with 15 ROIs taken for each sample.

Images were analyzed using custom LabVIEW 7.1 software to track and report the speed of each microtubule in a ROI. NI Vision 7.1 image segmentation algorithms via pattern matching identified microtubules. Tracking of microtubules was stopped if the microtubule end was within a few pixels from the boarder of the ROI or if a microtubule overlapped with another microtubule. If a microtubule had fewer than 100 consecutive image frames without tracking problems, the track was discarded. Microtubules that had a segmented area less than 55 pixels were also discarded. These values were determined empirically and were found to provide well-tracked microtubules with few tracking errors.

Tracking provided the x and y position of microtubule ends with sub-pixel accuracy. This time series data was smoothed using a Gaussian window with a standard deviation of 2 seconds in order to eliminate transverse Brownian noise. Smoothed data within 5 seconds of the beginning and end of a microtubule track were discarded to eliminate edge effects due to the Gaussian smoothing. After smoothing, the instantaneous speed of the microtubule was then calculated. Speed versus time data for all the microtubules in an individual ROI were then concatenated together and the most likely speed was extracted using a kernel density estimation (KDE) using a Gaussian kernel of width 50 nm/s [32]. Using a KDE method instead of determining a simple mean speed reduced our sensitivity to microtubule pausing, stalling, or tracking errors. The large kernel width also reduced the sensitivity to speed changes due to the number of kinesin molecules pulling on a microtubule seen by Gagliano *et al*. [33].

Results and Discussion

Results

Figure 1 shows speed variations using increasing amounts of heavy-hydrogen buffer in the motility solution. Each data point represents three independent measurements where the error bars are the standard error of the mean. Several hundred microtubules were tracked for each data point at a constant temperature of 33.1 ± 0.1ºC. The most likely speed using only the light water buffer was found to be 1016 ± 11nm/s. Successive increases to the amount of heavy-hydrogen buffer used in the motility solution caused the most likely speed measurement to decrease in a linear fashion. At the highest concentration of heavy-hydrogen buffer used, the most likely gliding speed was found to be 799 ± 8nm/s. This value is about 21% lower than the light water buffer control. The deuterium-depleted light water buffer is also represented in this figure. The most probable speed at which the microtubules moved at in the deuterium-depleted buffer was found to be 1015 ± 4nm/s.

Figure 2 shows the speed variations using increasing amounts of the heavy-oxygen buffer used in the motility solution. Again, each data point represents three independent measurements with several hundred microtubules tracked in the ROI and the error bars are again the standard error of the mean. In this instance, the light water buffer control gave a microtubule speed measurement of 1007 ± 4nm/s. At the highest concentration of heavy-oxygen buffer used, the

most likely speed was found to be 954 ± 16nm/s. This is approximately a 5% decrease in speed as opposed to the 21% decrease in speed measured using the heavy-hydrogen water isotope.

Figure 3 shows that heavy-hydrogen does not cause permanent damage to the kinesin-1 surface. Using the resealable flow cell, we initially observed microtubules gliding in a light water motility solution for our standard 15 regions. We then exchanged the light water motility solution with twice the volume of the sample cell with the heavy-hydrogen motility solution. We then resealed the flow cell with cellophane and observed microtubules in the heavy-hydrogen buffer. As can be seen in Figure 3, the average measured speed of microtubules dropped from 987 ± 2nm/s to 791 ± 1nm/s when the light water motility solution was exchanged with the heavy-hydrogen water motility solution. In order to ensure that heavy-hydrogen water did not damage the kinesin-1 surface irreversibly, another fluid exchange from the heavy-hydrogen motility solution back to the light water motility solution was done. Measured microtubule gliding speed values recovered back to 960 ± 2nm/s. Comparing the methods of sealing the flow cell with cellophane and nail polish indicates that using nail polish as the sealant does not interfere with motility. Nail polish contains organic solvents and other components that have the possibility to leach into the motility solution and could cause damage to the assay. However measured speed values using both nail polish and cellophane as the sealant show that stable speeds can be measured and that nail polish did not damage motility observations over the time span of our experiments.

Discussion

Adding light water buffer to the heavy-hydrogen buffer to measure speed variations due to $^{2}H_2O$ concentrations in the motility solution inevitably caused variations in the final pH of the solution. This was due to the fact that the light water buffer was pH-ed to a value of 6.89 while the pH of the heavy-hydrogen buffer was 7.30. In order for the two solutions to have the same effective pH, the heavy-hydrogen buffer should have been pH-ed to 6.48, which would have made the pD 6.89. Böhm *et al*. showed that pH does affect the gliding speed of microtubules [21]. However, they showed that speed increases with increasing pH and showed that pH values between 6.89-7.71 increased measured gliding speeds by approximately 50nm/s. Figure 1 shows that the speed difference from a solution containing all light water buffer (pH of 6.89) and one containing nearly all heavy-hydrogen buffer (pH of ~7.70) decreased measured speed values by approximately 200nm/s. Due to the fact that our measured speed values are of greater magnitude and in the opposite direction expected from pH increase indicates that the effect we see is due primarily to isotope exchange and not to pH variation. Obtaining the pH value of 7.30 also required the addition of more NaOH to solution. Doing so also increased the ionic strength of the solution, which Böhm showed should increase the gliding speed, which is again opposite to the effect we observed [21].

In light water, there exists approximately 17mM of deuterium in solution, which exist mostly as HOD molecules [34]. In order to determine if this small amount of deuterium in solution affected the gliding speed of microtubules, a motility solution that was made of deuterium-depleted light water was used. Every possible effort was made to ensure that the trace amounts of deuterium in this motility solution were as low as possible. We found that the gliding speed of microtubules using a deuterium-depleted buffer was 1015 ± 4nm/s. This value agrees with the intersection of the fit line in Figure 1 and was indistinguishable from light water speed measurements. We were unable to detect effects of deuterium-depletion in the gliding motility assay, as expected.

There exists a measurable drop in speed values using the resealable flow cell between the initial observations using the light water motility solution and the final observations using the light water motility solution (see Figure 3). This drop in measured speed values was attributed to

fluid exchanges in the resealable flow cell. Our initial experiments to determine the viability of a resealable flow cell used light water exclusively where we exchanged the fluid in the cell multiple times, see Text S2. Over successive fluid exchanges, the measured speed values decreased every time the fluid was exchanged and the resulting microtubule movement became untrackable after five fluid exchanges. The decrease in speed could be a result of kinesin and passivation being removed from the surface during successive fluid exchanges.

We observed that both heavy-hydrogen (^2H_2O) and heavy-oxygen water ($H_2{}^{18}O$) decrease the gliding speed of microtubules in a linear fashion. A similar decrease was observed for myosin and actin when substituting deuterium oxide for light water [8]. This indicates that both water isotopes affect a rate-limiting step for transport in the kinesin gliding assay. Kinesin bulk assays and single-motor assays suggest that the most rate-limiting step is inorganic phosphate release and/or kinesin rear head release [35-38]. The rate-limiting step in the gliding assay has not been explored as thoroughly, and it may be affected by surface-effects not observed in bulk or single-motor assays. We note that the proportional decrease in gliding speed is similar to the proportional reported increase in viscosity of the two pure isotopic solutions [39-40]. We did not attempt to measure the viscosity of any of our fractional mixtures.

Increased microviscosity could be the cause of our observed decrease in gliding speed, which could possibly slow the rate of inorganic phosphate release [41-42]. Investigating microviscosity requires further experimentation. However, if the effect we observed is due to microviscosity, then using water isotopes as a microviscosity probe could probe the effects of diffusion-controlled, rate-limiting steps in kinesin and other enzymes. We are also pursuing osmotic stress studies in the kinesin gliding assays [43]. Perturbing the osmotic pressure with osmolytes such as betaine, sucrose, or polyethylene glycol inevitably perturbs the viscosity as well. Thus, water isotopes, particularly $H_2{}^{18}O$ which has less effect on hydrogen bonding than ^2H_2O, may provide important complementary information for the osmotic stress studies.

Conclusions

We have shown that microtubule gliding speeds on kinesin surfaces is systematically slowed by increasing the amount of heavy-hydrogen, ^2H, or heavy-oxygen, ^{18}O, water isotopes in the motility solution. The relative effects of heavy-hydrogen versus heavy-oxygen are similar to the relative viscosity changes seen in either water type for which microviscosity may provide an explanation for the effect, however, further experimentation and theoretical work is necessary. Water isotopes appear to be an effective experimental knob that can be used to study the effects of water on kinesin activity. Moreover, changing the water isotope (particularly the heavy-oxygen substitution) could be an ideal method for perturbing the microviscosity of the solvent. Viscosity can be controlled to a certain degree by using different osmotic stress agents that affect viscosity differently. Some of the osmolytes we wish to study include; betaine, sucrose, and polyethylene glycol. If indeed the effects from heavy-oxygen water are due to microviscosity, the information in this article can be used in combination with osmolyte studies to isolate the effects of osmotic stress. Finally, some research groups are pursuing usage of molecular motor systems in microdevices [44-45]. It is feasible that heavy-hydrogen water is a superior solvent for these systems, due to its stabilizing properties, which include photostabilization [46]. The use of molecular motors in microdevices adds a further need for understanding the behavior of kinesin and microtubules in differing water isotopes.

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Figure 1. Microtubule gliding speed at 33.1 ± 0.1ºC versus concentration of ^{2}H in solution. *Solid circles* represent the mean of observations from three independent samples. Each of the three observations was an estimate of the most likely gliding speed of hundreds of microtubules in the sample after temperature equilibrium was reached (see text). *Error Bars* represent the standard error of the mean of the three observations. The *solid line* is to guide the eye and does not represent a fit of a theoretical model. The steady decrease in speed (as opposed to a roll-off at intermediate concentration) indicates that the added deuterium affects a rate-limiting step for microtubule gliding speed on kinesin-1 surfaces. Also shown is the gliding speed in deuterium-depleted water (1015 +- 4 nm/s), which was indistinguishable from light water (1016 +/- 11nm/s).

Figure 2. Microtubule gliding speed at 33.1 ± 0.1ºC versus concentration of ^{18}O in solution. *Solid circles* represent the mean of observations from three independent samples. Each of the three observations was an estimate of the most likely gliding speed of many microtubules in the sample after temperature equilibrium was reached (see text). *Error Bars* represent the standard error of the mean of the three observations. The *solid line* is to guide the eye and does not represent a fit of a theoretical model. As in Figure 1, the steady decrease in speed (as opposed to a roll-off at intermediate concentration) indicates that the added ^{18}O affects a rate-limiting step for microtubule gliding speed on kinesin-1 surfaces.

Figure 3. Microtubule gliding speed at 33.1 ± 0.1ºC versus time for a single sample with three different gliding buffers. Time is measured relative to the flow-in of the first motility buffer. Each *solid circle* represents the most likely gliding speed from hundreds of microtubules in one region of observation over a two-minute period. Error bars have been removed for clarity. Each set of measurements reveals the initial time necessary for the sample temperature to obtain equilibrium with the objective temperature. The first set of data represents observations with the light water buffer. An average speed of 987 ± 2nm/s was measured. The middle set of data represents observations after replacing the light water buffer with twice the volume of the flow cell with the heavy-hydrogen buffer. The measured average speed was 791 ± 1nm/s. The final set of data represents observations after re-filling the flow cell with twice the sample volume of light water buffer. The average measured speed was 960 ± 2nm/s. The return to nearly the same speed as the first data set indicates that heavy water does not cause severe permanent changes to the kinesin surfaces.

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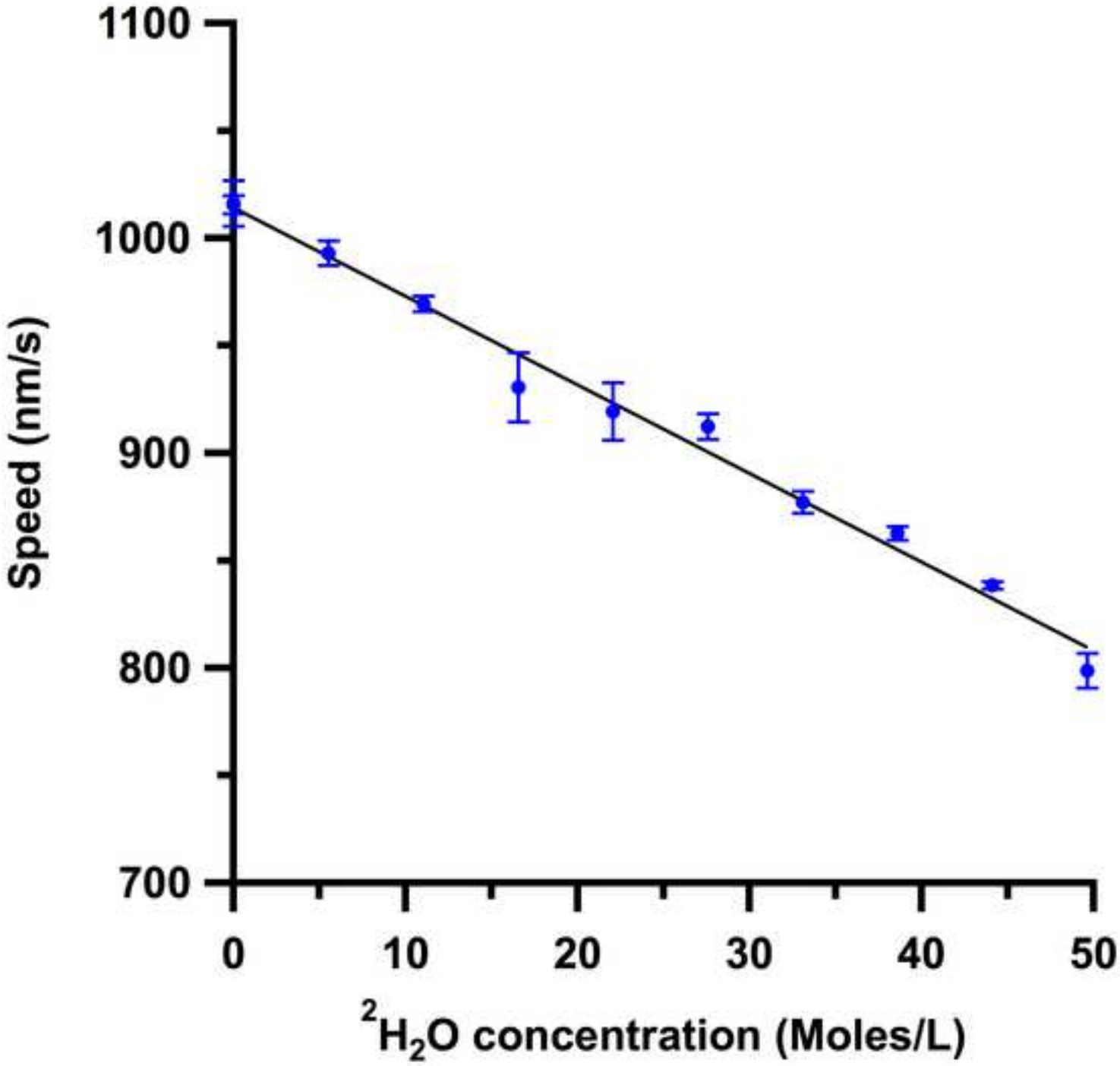

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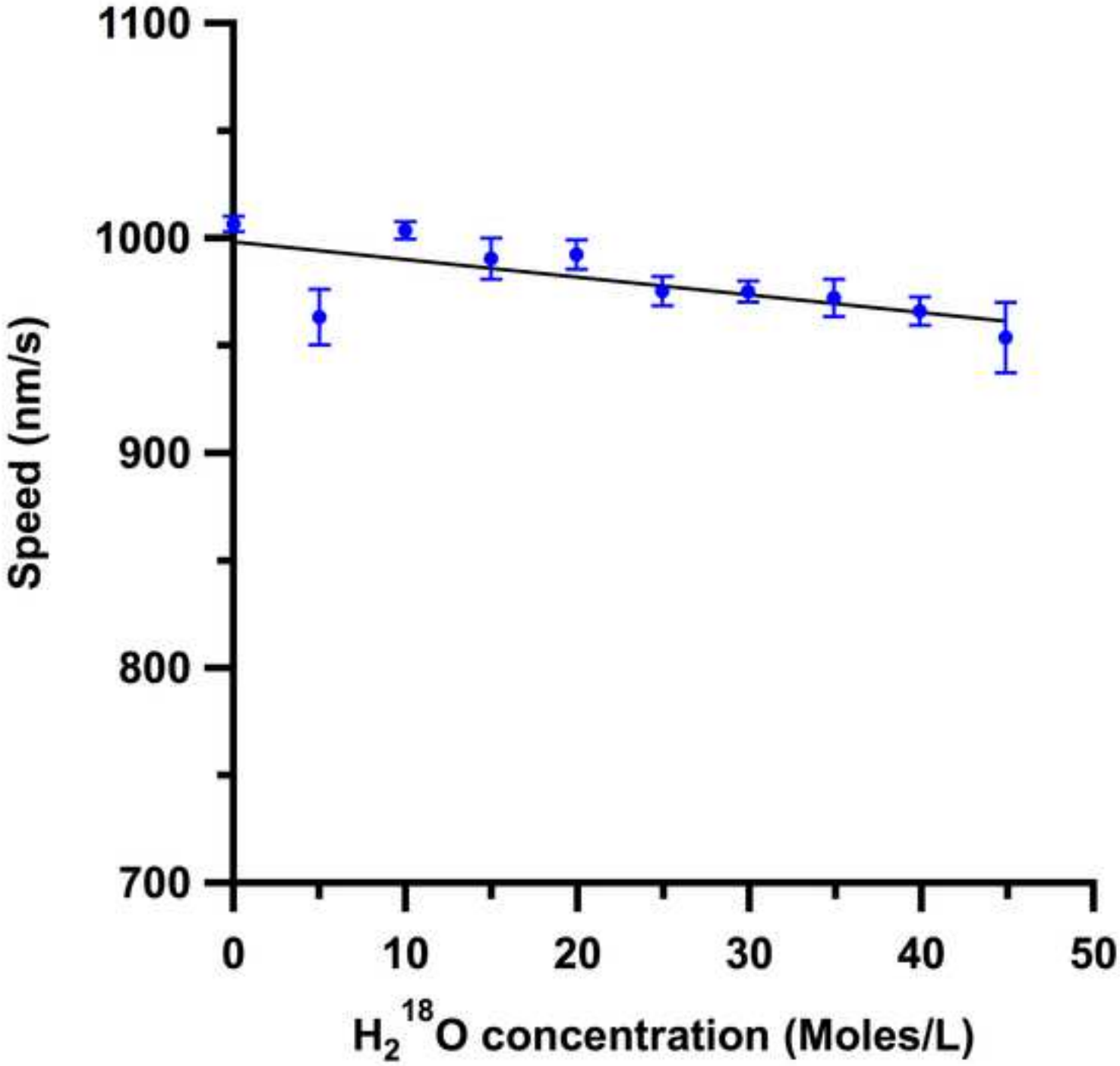

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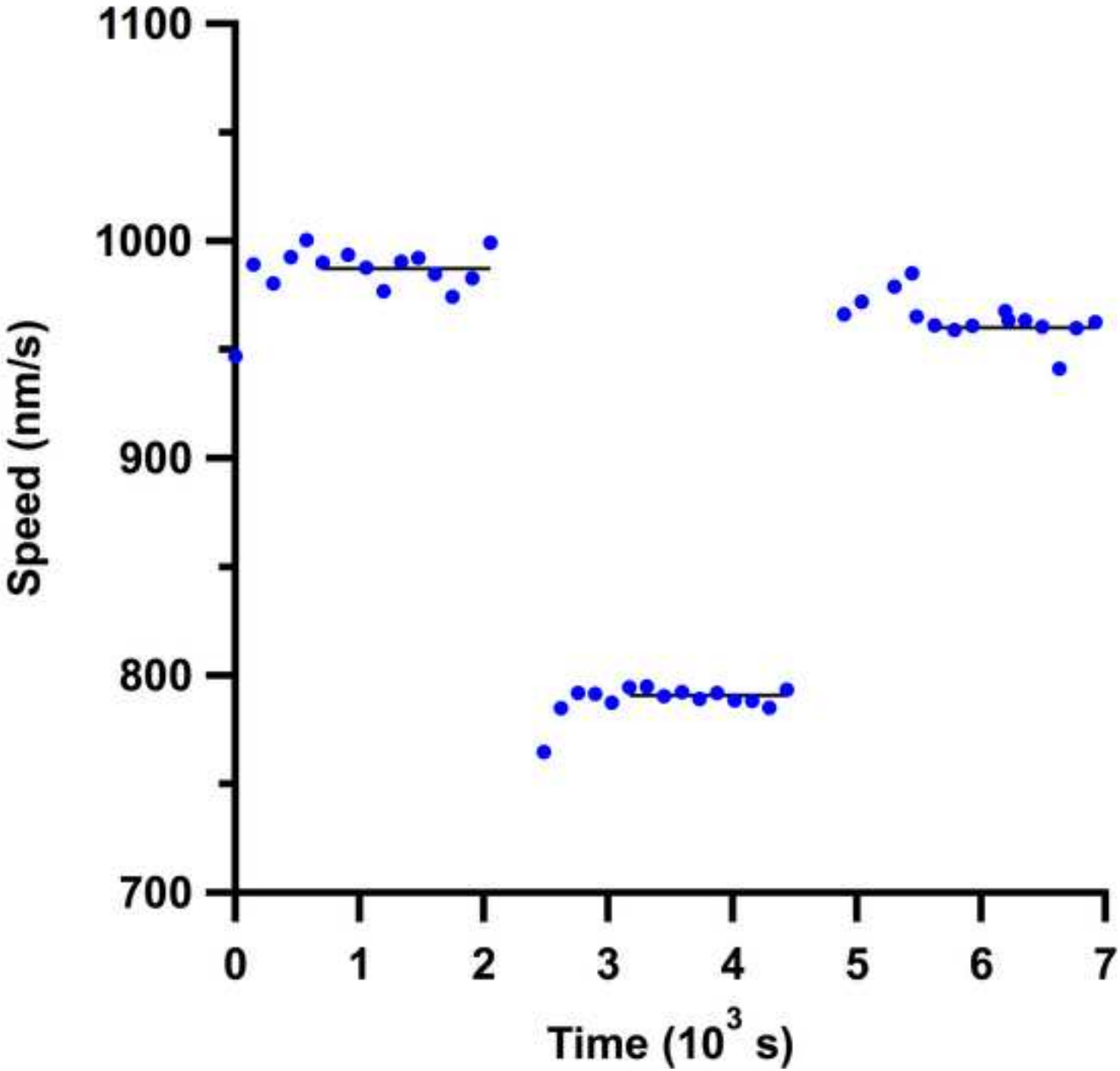

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