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Temperature control device for single molecule measurements using the atomic force microscope

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The design and implementation of a temperature control device for the atomic force microscope (AFM) are described. This device is based on a thermoelectric module which can be used for both heating and cooling the samples in the AFM liquid chamber within the range of 5–50 °C. A thermocouple is inserted in the liquid chamber to measure the sample temperature. A commercial thermoelectric temperature controller is used to keep the temperature constant during a measurement, which can be stabilized within 0.1 °C. To dissipate the heat generated by the thermoelectric module during cooling, a water cooled heat sink is used. Using this device, imaging and mechanical unfolding experiments were carried out at different temperatures. The results show that the temperature control device does not significantly reduce the imaging capacities of the AFM, and that the force-induced unfolding of individual protein molecules can be readily observed at different temperatures. Temperature dependent single molecule measurements can broaden the applications of AFM and reveal new insights into the macromolecular structures and processes.
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INTRODUCTION

The atomic force microscope (AFM) has been widely used in experimental research in biophysics due to its unique capabilities to obtain three-dimensional images of biomolecules with high resolutions in physiologically relevant buffers and to detect the weak interaction forces between individual macromolecules. For example, AFM has been used to follow the dynamic process of protein binding to DNA,[1] and AFM force measurement has directly determined the base pairing forces of DNA molecules. AFM has also been used to measure solution viscosity by monitoring the power spectrum of AFM cantilever fluctuations.[2] In the past several years, significant new insights have been obtained on the process of protein folding and unfolding by using the AFM to investigate the mechanical force induced unfolding and refolding of individual protein molecules.[3–12] The study of the force induced unfolding of the immunoglobulin (Ig) domains of titin, a giant sarcomeric protein of striated muscle, using the AFM revealed the molecular mechanisms of passive force generation in muscles.[3] The mechanical stability and kinetic properties of tandem spectrin repeat,[4] T4 lysozyme,[5] ubiquitin, vascular cell adhesion molecule-1 (VCAM-1),[13] and the Ig domains of a cell adhesion molecules,[14] have also been investigated by this approach. The folding trajectory of ubiquitin molecules was characterized with the AFM operation in the constant force mode, revealing that the folding process did not simply follow a two state model, but involved a more complicated series of conformation changes.[12] Additional information can be obtained about the systems under study if more variables can be controlled and varied in these experiments.

To increase the information content of AFM measurements, various instrumental improvements have been attempted. One of these is to add the capability to control the sample temperature, since temperature is an important parameter often used in experimental research in biophysics, biochemistry as well as material sciences. Temperature dependent measurements can provide important information on processes such as phase transitions and macromolecular reactions in microscopy experiments. The structures of biological molecules and other materials, as well as the processes of biomolecular binding can be modulated by temperature.[15] For example, the temperature-induced surface roughness change of NiTi shaped memory thin films was measured by temperature-controlled AFM,[16] and the phase transition temperature was then determined. The phase transition from the L_α to P_β phase of dimyristoylphosphatidylcholine (DMPC) was studied by temperature-controlled AFM at a high temperature resolution (0.1 °C).[17] The dynamic process of phase transition of poly[(*R*)-3-hydroxybutyrate (P3HB) single crystals was studied within a temperature range of 25–140 °C.[18] Recently temperature dependent experiments on force-induced protein unfolding were carried out, in which spectrin repeats were mechanically unfolded with the AFM at several temperatures from 10 to 42 °C.[9] The results showed that temperature induced-structural changes induced shifts in unfolding pathways.[19] An estimation of the energy landscape roughness of a protein complex was accomplished by measuring the temperature dependent unbinding forces with a commercial AFM.[19]

Various methods for temperature control have been reported for different purposes. Cryogenic atomic force

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microscopes[20–24] (cryoAFMs) were developed to operate at temperature as low as a few kelvins to study tip-gated conductance of a GaAs high electron mobility transistor device and to image biological samples[22] with high spatial resolutions. A combined AFM and high resolution optical microscope, which could simultaneously acquire force and optical images over the temperature range (4–300 K), was reported.[25] In the design, cooling was provided by a liquid helium flow cryostat and the microscope was cylindrical with a height of 14.5 cm and a diameter of 3.55 cm to fit in the cryostat. A hydrothermal AFM which could perform imaging in aqueous solutions at temperatures up to 150 °C and six atmospheric pressures was reported,[26] where a resistively heated ceramic booster heater was used to maintain the temperature of the solution. In the temperature dependent protein unfolding studies,[9] the low temperature was achieved by operating the AFM in a cold room. Generally speaking, it is easier to design and build heating stages for AFM to perform temperature dependent experiments above room temperature. There are commercially available heating stages for various AFM models.[15,27] It is more complicated to design an effective and user friendly cooling stages for AFMs. There have been only a limited number of reports on such devices. A variable temperature fluid stage that could work from −5 to 130 °C was reported by Workman and Manne.[28] This stage, with a size of $5\times5\times2$ cm^3, was based on a thermoelectric (Peltier) module and used with a Dimension 3100 AFM (Veeco/Digital Instruments, Santa Barbara, CA). Another temperature control method for AFM experiments was reported by Hegner *et al.*[29] In this approach, a fluid cell was built in such a way that the enclosed buffer solution was in direct contact with a Peltier element which controlled the temperature of the solution. This design was used to investigate the temperature dependence of the unbinding forces between complementary DNA strands.[30] Peltier device based AFM stages have also been developed commercially for several specific models.[15,27]

Our goal was to have a convenient and efficient temperature control device for the AFM to carry out single molecule experiments in the temperature range most relevant to biological samples. The commercial products do not meet all of our needs. Therefore we designed and built a temperature control device that works well for the temperatures at which water is in the liquid phase. The device is based on a small Peltier module and a microthermocouple, and it can control the sample temperature within the range of 0 to 50 °C with a precision of 0.1 °C. To test the performance of the temperature control stage, imaging and protein unfolding experiments were carried out at different temperatures using a multimode Nanoscope IIIa AFM (Veeco/Digital Instruments, Santa Barbara, CA).

INSTRUMENTATION

Instrumental design

The main difficulties of building the temperature control devices are the high mechanical and temperature stability requirements of AFM measurements, the continuous change of temperatures from above room temperature to below room temperature, and the space limitations of the AFMs. The device needs to keep the temperature stable for an extended period of time, and also be able to change the sample temperature in a time scale of several minutes. For many AFM models, the space is limited for adding extra components, which restricts the choices of cooling methods and interferes with the heat dissipation. Mechanical vibrations are detrimental to AFM measurements; therefore fans or pumps cannot be utilized. A thermoelectric (Peltier) module was selected as the heating/cooling element for the device since it can provide continuous cooling and heating without moving parts. Figure 1 shows the overview of the temperature control device mounted on the AFM with a liquid chamber. Gold coated glass cover slip, onto which sample is deposited, is mounted on the Peltier device using thermal grease (TG-001, Melcor, Trenton, NJ). Good thermal contact between the cover slip and the Peltier surface is critical for efficient cooling and heating, and in the meantime, a stable bond between the two surfaces is necessary for AFM operation. It was found that this thermal grease satisfies both requirements after testing several different types.

The Peltier module used in the device was purchased

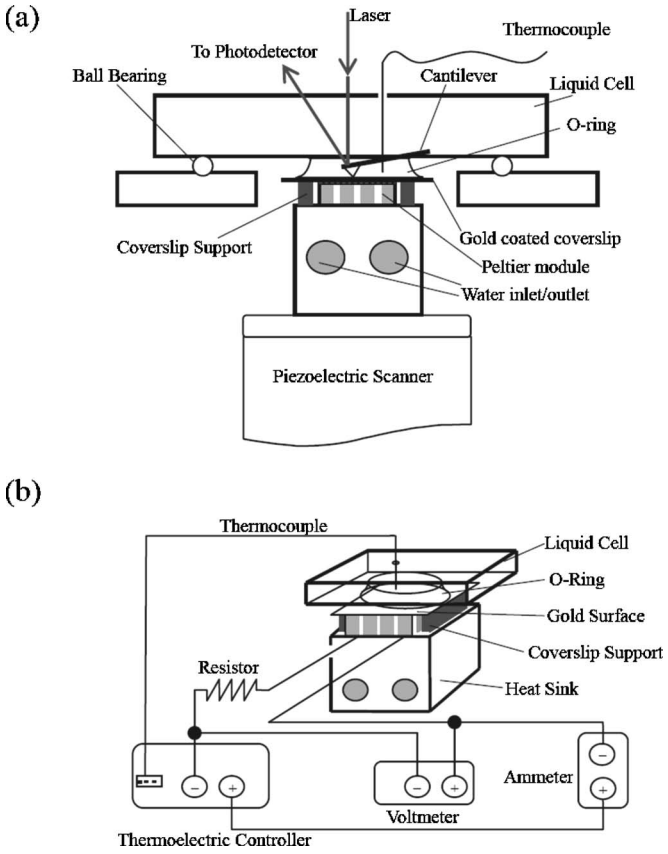

FIG. 1. Schematic of the temperature control system fitted to an AFM scanner and a liquid chamber, which is positioned inside the AFM head. (a) A side view of the temperature control device. The liquid cell is supported by the three ball bearings in the AFM head, and the piezoelectric scanner can move the sample (deposited on the gold surface) in three dimensions with respect to the AFM tip. The o-ring (6 mm in diameter) seals ~50 μl of liquid in which the cantilever and the thermocouple are immersed. Flexible tubes (3.6 mm in diameter) are connected to the inlet and outlet of the heat sink to pass the cooling water. (b) The electrical connections of the temperature control system.

from Melcor (model MTCA hot2.0-18-F2A, Trenton, NJ 08648), with dimensions of $6.0\times7.2\times2.2$ mm^3. This module was chosen because it has a surface area that matches the bottom surface area of the liquid chamber (enclosed by an o-ring) thus providing the most efficient control, while larger modules caused longer delay times for temperature changes and required larger heat sink for cooling operations. A commercial thermoelectric controller (model MTCA 6040, from Melcor, Trenton, NJ) and a microthermocouple (Chromega™-Constantan E-type, 125 μm diameter, from Omega Engineering, Inc. Stamford, CT) were used for controlling the sample temperature.

When the system works in the cooling mode, a water cooled heat sink (made of brass, with dimensions of $1\times1\times1$ cm^3) is used to dissipate heat from the hot surface of the Peltier device, as shown in Fig. 1. The Peltier module is fixed on the heat sink by thermal epoxy (TEC-002, Melcor, Trenton, NJ 08648), and a 12 mm diameter steel disk is glued by super glue (Super Glue Corporation, Pacer Technology, Rancho Cucamonga, CA) to the bottom of the heat sink. Two flexible tubes [inside diameter (I.d.)=1.8 mm and outside diameter (o.d.)=3.6 mm] are used to pass water through the heat sink. Cold water is drawn from a large container through siphoning. Experiments showed that the water flowing did not introduce a significant amount of noise to the measurement results (see Fig. 4). A stable temperature of 5 °C could be achieved with a water flow rate of 0.03 ml/s. When working at temperature above 15 °C, water cooling was not needed. To assemble the temperature control system, the protein sample is deposited on the gold coated glass cover slip, which is then attached to the surface of the Peltier device via thermal grease (TG-001, Melcor, Trenton, NJ). Since the glass cover slip is larger than the surface of Peltier device, a supporting feature was added on the top surface of the heat sink to prevent tilting of the cover slip when the o-ring is pressed on it, as shown in Figs. 1(a) and 1(b). Digital multimeters are used to monitor the voltage and current applied to the Peltier module, and a resistor is used for overload protection.

Sample preparation and AFM measurements

Ubiquitin molecules are used for the protein unfolding experiments. The ubiquitin molecules are connected into polymers that have been synthesized via a protein engineering approach.[6] In the pulling experiments, the ubiquitin molecules unfold one by one, each unfolding event generates a peak in the force-extension curve. The experimental procedures were described in detail elsewhere.[6] Briefly, the protein was dissolved in phosphate-buffered saline (PBS) buffer with a protein concentration of 50 μg/mol, and ~20 μl of the protein solution was deposited on a fresh gold surface. A LABVIEW (National Instruments, Austin, TX) program was used to control the movements of the AFM cantilever, whose spring constant was known through a calibration procedure. The tip was first pushed onto the sample surface with a force of a few nanonewtons for 5 s to allow the molecules to interact with and attach to the tip. Then the tip was retracted from the surface with a specified pulling speed. The force exerted on protein polymer was then measured as a function of the relative extension. The cantilevers used in the experiments were triangular Si_3N_4 cantilevers (model MLCT-AUHW, cantilever A), purchased from Veeco Metrololgy (Santa Barbara, CA), with a nominal spring constant of 50 pN/nm.

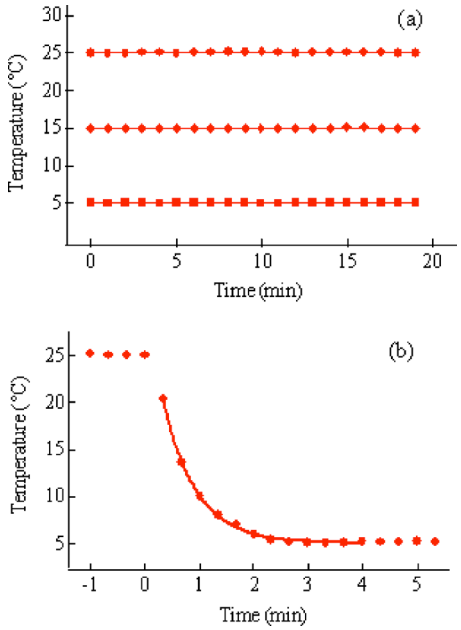

FIG. 2. (Color online) Performance of the temperature control device. (a) Stability of the sample temperature. The data show that when the set temperatures were 5, 15, and 25 °C, respectively, the sample temperature was stabilized at averages of 5.1 (squares), 14.9 (diamonds), and 25.1 °C (circles), respectively, with fluctuations less than 0.1 °C. The solid lines are the average values of the measured temperatures. (b) The rate of temperature change after lowering the set temperature. At $t<0$, the temperature was stable at 25 °C. At $t=0$, the set temperature was changed to 5 °C, and the sample temperature was measured every 20 s (filled circles). The decreasing part of the curve can be fitted well with an exponential function $T=T_1+(T_0-T_1)\exp(-t/\tau)$, with $\tau=36$ s (solid line).

EXPERIMENTAL RESULTS AND DISCUSSION

Performance of the temperature control device

Using the Nanoscope IIIa AFM (Veeco/Digital Instruments, Santa Barbara, CA), the temperature stability and the heating/cooling rates of the temperature control device were determined. The temperature controller was set to dual mode which could go continuously from cooling to heating. Figure 2(a) shows the temperature of the sample as a function of

TABLE I. Temperature response time constants when the set temperature was changed from one value (T_0) to another (T_1). The values were obtained from fitting the data to an exponential function as shown in Fig. 2. The time is shown in seconds.

	T_1 (°C)				
T_0 (°C)	5	10	15	20	25
5	N/A	12	17	22	29
10	18	N/A	12	17	23
15	23	20	N/A	13	17
20	29	24	16	N/ A	14
25	36	28	21	15	N/A

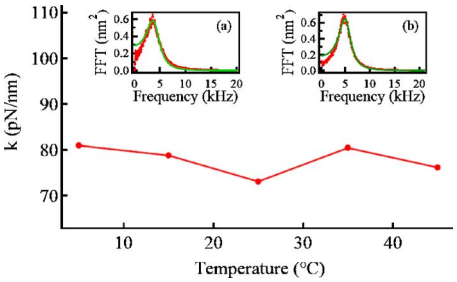

FIG. 3. (Color online) The measured force constant of an AFM cantilever at different temperatures. The force constant was determined by recording the thermal vibrations of the cantilever and applying the equipartition theorem (Ref. 31). Insets: The power spectrum of the cantilever's thermal vibrations at (a) 5 °C and (b) 45 °C. The solid curves are fitting of the data to the power spectrums of simple harmonic oscillators (Ref. 33).

time for three temperature settings. The temperature fluctuations are less than 0.1 °C in both cases. Another important parameter of the device is the rate at which the sample can be cooled or heated. Figure 2(b) shows the temperature as a function of time after the set temperature was changed from 25 to 5 °C. The curve can be approximately fitted to an exponential function, $T=T_1+(T_0-T_1)\exp(-t/\tau)$, with $\tau=36$ s. Table I lists the response time constants (τ) measured for several different temperature changes. Since spring constants of the cantilevers directly affect the measured force values, each cantilever used in the experiments was calibrated before and after an experiment using the energy equipartition method.[31] Cantilevers were also calibrated at different temperatures to see if the spring constant was influenced by temperature change. Figure 3 shows the spring constant of a cantilever measured at several different temperatures, as well as the thermal vibration power spectrums at two temperatures. These results indicate that the spring constants of the cantilevers are not significantly affected by temperature changes in this temperature range.

Imaging of sputtered gold surface

To determine the ability to perform high resolution imaging at different temperatures using the device, sputtered gold surfaces were imaged with the AFM. The gold film was first sputtered on a freshly cleaved mica surface, and then gold coated mica was glued to a glass cover slip with epoxy. A fresh and flat gold surface was obtained after the mica had been peeled off.[5,6] The imaging was carried out in water at various temperatures. As shown in Fig. 4, similar nanometer sized features were observed at different temperatures, indicating that the temperature control device did not significantly reduce the imaging capacities of the AFM.

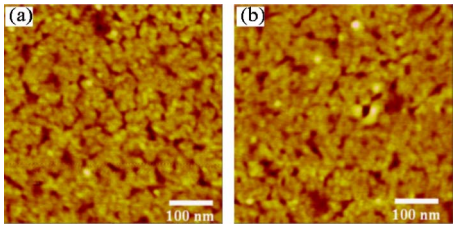

FIG. 4. (Color online) AFM images of sputtered gold surface obtained at (a) 5 °C and (b) 25 °C. The images (not the same area of the sample) were acquired in pure water using the tapping mode (cantilever resonant frequency=9.35 kHz). Both images have a size of 500×500 nm^2.

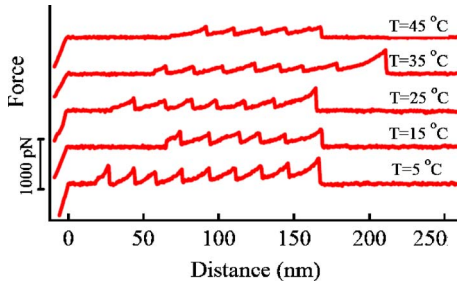

FIG. 5. (Color online) Force vs distance curves from mechanical unfolding of polymeric ubiquitin molecules obtained at different temperatures. In the experiments, the ubiquitin octamer could be tethered between the surface and the tip at any two points on the chain, thus the number of unfolding events observed was different for each pulling. The level portion on the right side of each curve corresponds to zero force where the tethered polymer chain detached from the tip or the sample. The pulling speed was 1000 nm/s.

Mechanical unfolding of ubiquitin molecules

Figure 5 shows several forces versus distance curves from mechanical unfolding of ubiquitin molecules at different temperatures. The peaks in these curves represent the unfolding events of individual ubiquitin molecules. As can be seen from the figure, with the temperature control device operating, the unfolding forces of the protein molecules could still be readily determined from the force curves. The dependence of the unfolding forces on temperature is plotted in Fig. 6 which includes data acquired at two different pulling speeds. Each point in the graph is the average of a number of data points (see figure caption). Shown in Fig. 6 are also the distributions of the unfolding forces measured at two temperatures. These data show that the unfolding forces of ubiquitin have considerable fluctuations, a large part of which is due to the nature of the single molecule measurements, as suggested by Monte Carlo simulations.[6] According to a recent theory, the temperature dependence of the unfold-

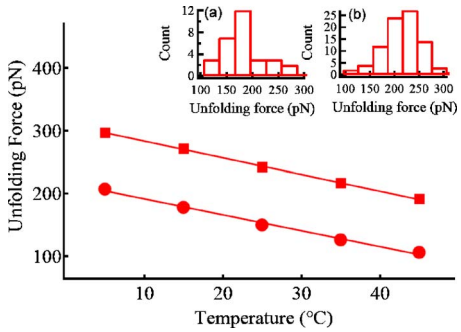

FIG. 6. (Color online) The temperature dependence of the unfolding forces of ubiquitin molecules at pulling speeds $v=50$ nm/s (filled circles) and $v=1000$ nm/s (filled squares). The unfolding forces decrease nearly linearly with temperature, as shown by the linear fits to the data (solid lines). Each point in the plot is the average of n force values obtained from the force curves (see Fig. 5). For $v=50$ nm/s, $n=38$, 30, 43, 21, and 6, respectively, for the temperatures of 5, 15, 25, 35, and 45 °C. Similarly, $n=79$, 63, 62, 87, and 56 for $v=1000$ nm/s. Insets: Histograms of the measured force values. (a) $v=50$ nm/s, T=15 °C and (b) $v=1000$ nm/s, T=35 °C.

ing forces can provide information about the folding free energy landscape of protein molecules, which will help us to solve the protein folding problem.[19,32]

DISCUSSION

A flexible and efficient temperature control device was designed and built for performing temperature dependent measurements with AFM. The sample temperature could be controlled within the range of 5–50 °C at a precision of 0.1 °C. Imaging experiments have shown that the temperature control device does not significantly reduce resolution of the AFM. Mechanical unfolding experiments on single ubiquitin molecules were carried out as a function of temperature, and the unfolding force of protein molecules was observed to increase with decreasing temperatures. Temperature control increases the information content of AFM single molecule experiments and will be able to provide important information on protein folding mechanisms and other biophysical processes.

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