LabVIEW-operated novel nanoliter osmometer for ice binding protein investigations

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Short Abstract:

Ice binding proteins (IBPs) inhibit ice growth and are a promising additive for use in the cryopreservation of tissues. The main tool used to investigate IBPs is the nanoliter osmometer. We developed a home-designed cooling stage controlled by custom-made LabVIEW software. The described apparatus enables us to manipulate the temperature of the sample in an ultra-sensitive manner.

Long Abstract:

Ice-binding proteins (IBPs) protect cold-environment organisms by interacting with extracellular ice crystals. These proteins have been found in fish (1), plants (2), insects (3), fungi (4), and bacteria (5). IBPs adsorb to the surfaces of ice crystals and lower the temperature at which the ice crystals grow, thereby creating a gap (thermal hysteresis [TH]) between the melting point and the nonequilibrium freezing point, within which ice growth is arrested, (6-8) see Fig 1. One of the main tools used in IBP research is the nanoliter osmometer, which permits measurements of the TH activities of IBP solutions.

We designed a custom-made computer-controlled nanoliter osmometer system using a LabVIEW platform (National Instruments). The cold stage described previously (7, 8) contains thermoelectric coolers driven by commercial temperature controller (Newport 3040 or 3150) that can be controlled via LabVIEW modules. The major advantage of this system is its sensitive temperature control (0.002°C). Automated temperature control enables a fixed temperature ramp coordinated with a video output containing the experimental details. We tested a 58 kDa hyperactive IBP from the Antarctic bacterium *Marinomonas primoryensis* (*Mp*IBP) (9) tagged with eGFP, constructed by Peter Davies' group (Queens University), to study the time dependence of the TH activity. We showed that the temperature change profile affected the TH activity. Excellent control over the temperature profile in these experiments significantly improved the TH measurements. Simultaneous real-time video analysis of the images offered additional control options during the experiments' procedures (10). A stage for the inverted microscope was developed to accommodate the use of temperature-controlled microfluidic devices (11).

Protocol Text:

1.) Sample preparation

Preliminary procedures:

- **Glass capillary for solution injection -** Using a capillary puller (Narishige, Tokyo, Japan) prepare a sharp pipette from a glass capillary tube (Brand GMBH, Wertheim, Germany). The end product should be a capillary tube with a fine opening. To verify the size of the opening, pass air through the capillary to obtain fine bubbling in clean water. The capillary should be almost blocked but sufficiently open to allow sub-millimeter bubbles to form.
- **Copper disc cleaning –** Sonicate the copper discs for 10 minutes in 0.1% Micro-90 soap (Cole-Parmer, Vernon Hills, Illinois, USA), then wash with DDW. Introduce the copper discs into an isopropanol (technical) solution and sonicate again for 10 minutes. Finally, dry the copper disc with filtered air.
- **Double layer cover glass assembly –** Following are instructions for preparing a cover glass assembly that allows observation without condensing moisture on the cover glass surface. Place one Drierite (W.A. Hammond Drierite, Xenia, Ohio, USA) particle on a 22X22 mm coverslip. Using a hot glue gun, place a thin layer of glue on the edges of the coverslip, surrounding the Drierite particle. Upon formation of a complete circle, quickly cover the glue layer with a second coverslip, forming a sandwiched layer of glue with a Drierite particle in it. This will prevent condensation that might block the view when the sample is cooled to low temperatures.

1.1) Place 3-4 µL immersion oil B (Cargille laboratories, Cedar Grove, New Jersey, USA) on the back side of a 7 mm copper disc having 500 µm holes through the disc.

1.2) Position the copper disc on the cooling stage with the immersion oil side facing down.

1.3) Connect the capillary tube (from the blunt edge) to a rubber tube, which is connected to a glass syringe.

1.4) Before using the capillary tube, check that the opening at the sharp edge is in an appropriate size (see the Preliminary procedures).

1.5) Slowly insert the glass capillary into the prepared IBP protein sample tube (*Mp*IBP-GFP 2.4 µM in 20 mM $CaCl_2$ and 25 mM Tris-HCl at pH 8) and pull the glass syringe until the glass capillary contains 0.1 µL of the protein solution.

1.6) Insert the sharp edge of the glass capillary (containing the protein solution) into one of the holes in the copper disc on the cooling stage.

1.7) Observing through the microscope (Olympus, Tokyo, Japan, 10X objective), carefully penetrate the immersion oil layer with the glass capillary tip and press (very delicately) on the glass syringe to pipette out a small amount (~10 nL) of the protein solution, creating a 200 µm size droplet.

1.8) Cover the hole in the cooling stage with the double layer cover glass assembly (see the Preliminary procedures).

2.) Cooling stage set-up

2.1) Connect the cooling stage to the inlet and outlet water pipes, and connect the inlet pipe to a water pump.

2.2) Connect a dry air pipe to the inlet in the cooling stage. The dry air flows through a Drierite column that is in-line with the air pump and hosing.

2.3) After all pipes are connected to the cooling stage, operate both pumps (air and water). Note that the cooling elements should not run without a heat sink.

2.4) Turn on the temperature controller and camera, followed by the LabVIEW program.

3.) **TH activity measurement**

3.1) Begin video recording.

3.2) Press the cooling button and set the temperature to –40°C.

3.3) Initially, the solution droplet is clear. At low temperatures, typically –35°C, the droplet changes color, indicating that the solution has frozen. Immediately after the sample has frozen, increase the temperature slowly until the bulk ice begins to melt.

3.4) Form a single ice crystal (10 µm in size), and note its melting point (the highest temperature at which melting has ceased). At this point, one might optionally switch to 50X magnification (Nikon, Tokyo, Japan).

3.5) Set the temperature about 0.04°C below the melting point of the crystal and begin a temperature ramp with a 10 minutes delay. Adjust the rate of the ramp to the desired rate. During this time, the crystal will be exposed to the IBPs.

3.6) Upon completion of the 10 minutes exposure time, the temperature will decrease automatically.

3.7) Observe the crystal during this temperature decrease period, noting the temperature at which a sudden burst of growth occurs. Write down the crystal burst temperature.

3.8) The difference between the melting point and the freezing point (crystal burst temperature) is the thermal hysteresis activity of the IBP solution.

4.) Measurement of the time-dependent TH activity

4.1) Follow the protocol described in Sections 3.1–3.4.

4.2.) After formation of the crystal, set the delay time of the ramp as desired, and turn on the ramp.

4.3) The temperature will decrease at a fixed rate (according to the operators' needs) exactly and automatically once the ramp delay time has passed.

4.4) Document the temperature at which the crystal burst occurs. This is used to calculate the TH activity for the time between crystal formation and the crystal burst.

Representative Results: Measurement of the kinetics of IBPs.

The LabVIEW-operated nanoliter osmometer facilitates the performance of accurate TH activity measurements. A fixed temperature decrease rate also permitted measurement of the kinetics of IBP adsorption onto ice. The precise temperature control enabled by the nanoliter osmometer was crucial for these experiments. Exposure time of an ice crystal to IBPs in the solution is defined as the time period from the formation of the crystal (the end of the melting process) until the sudden growth of ice around the crystal (crystal burst). We found that exposure time of the ice crystals to the IBPs was crucial for the TH activity. Short periods of IBP exposure time (a few seconds) produced low TH activity in the *Mp*IBP-GFP solution (2.4 µM) (Fig 3). The TH activity increased with IBP exposure time until it reached a plateau at 4 minutes IBP exposure. At higher IBP concentrations, the plateau was reached at shorter times.

Tables and Figures:

Figure 1 – Schematic diagram showing IBPs adsorbed to ice (8).

Figure 2 – Screenshot of the LabVIEW interface.

Figure 3 –*Mp*IBP TH activity as a function of ice crystal exposure time to IBP.

Discussion: This work demonstrates a computer-controlled nanoliter osmometer that enables accurate measurements of TH activity with extraordinary temperature control. In any temperature-sensitive system, unwanted temperature gradients must be avoided. To avoid temperature gradients in the apparatus presented here, the test solution droplet must be positioned in the center of a hole in the copper disc cooling stage (step 1.7). Additionally, the single crystal should be in the center of the droplet rather than around the edges (in most cases, this will happen spontaneously). An inverted microscope stage was developed here, and the microfluidic devices were temperature-manipulated to enable solution exchange experiments involving ice crystals and GFP-tagged IBPs. The LabVIEW-controlled system was also been adapted to a Clifton stage by connecting the 3040 temperature controller via a designated adapting electric circuit. Such a system is operating in the Davies' lab (12). The LabVIEW software is available upon request.

In conclusion, we have described a nanoliter osmometer that enables the sensitive control and manipulation of the temperature, control over the rate of temperature increase/decrease (with 0.002°C sensitivity), and enables a video interface embedded in the LabVIEW program for real-time analysis. This system is capable of performing reproducible rate-controlled experiments that are important for investigating the kinetics of IBPs interacting with ice. Such experiments can address several long-debated issues surrounding the mechanism of action of IBPs.

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Table of specific reagents and equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
Immersion oil Type B	Cargille laboratories	16484	
Drierite	W.A. Hammond Drierite	043063 2270g	
Glass capillary tubes	Brand GNBH	7493 21	75 mm long, 1.15 diameter
Temperature controller	Newport		Model 3040
Light microscope	Olympus		Model BH2
10X objective	Olympus		Splan 10, 0.3, 160/0.17
50X objective	Nikon		CF plan, 50X/0.55 EPI ELWD
CCD Camera	Provideo	cvc-140	
Capillary puller	Narishige		
Micro 90 cleaning solution	Cole-Parmer	EW-18100-10	

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