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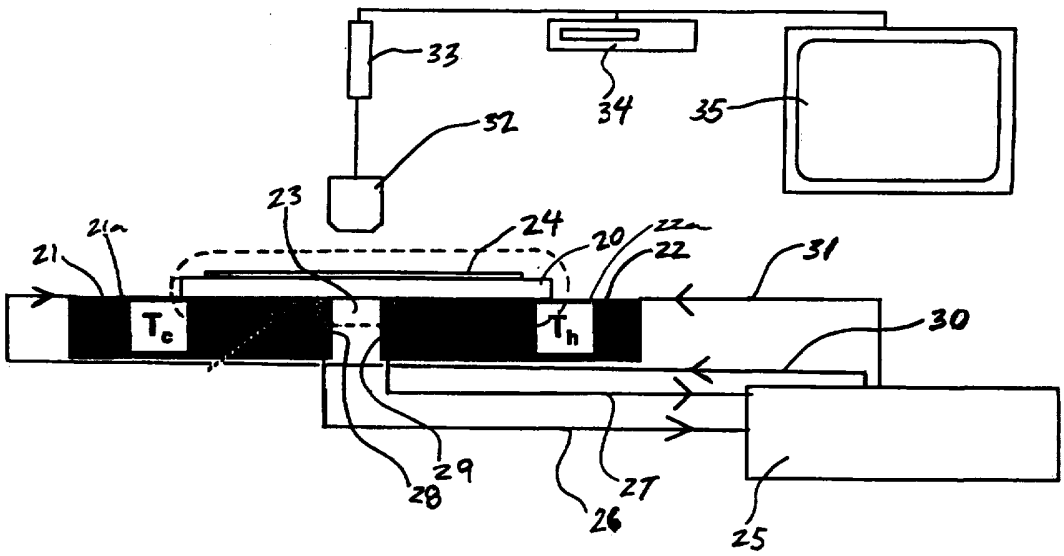

(57) Abstract

A thermal-gradient osmometer includes a substrate (20) spanning two independently variable temperature zones (21, 22) separated by a gap (23), and a plurality of sample rows (24a-24d) provided on or in the substrate (20). The zones (21, 22) establish a thermal gradient across the substrate (20). In operation, an unknown solution whose phase transition temperature is desired is provided in a first sample row. Next, a first and a second solution having respectively, higher and lower phase transition temperatures than the unknown solution are placed in a second and third sample rows, respectively. The three sample rows are then exposed to the thermal gradient so as to cause a portion of the three solutions to undergo a phase transformation while generating respective first, second and third solid-liquid interfaces in their respective sample rows. The phase transition temperature of the unknown solution is determined based on the position of the third interface relative to the first and second interfaces.

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TEMPERATURE-GRADIENT OSMOMETRY

DESCRIPTION

1. Field of the Invention

The present invention relates to a freezing/melting point osmometry device and methods to perform freezing point osmometry. In this context, the invention is referred to as temperature gradient osmometry (TGO). In addition, the device and methods find application in the fields of determining the compositions of materials, such as alloys, and also in zone purification processes.

2. Background of the Invention

The freezing temperature depression of a solution is a thermodynamic property indicative of the solvent free energy and is considered colligative because equal numbers of chemically active particles per mass of solvent produce equal freezing temperature depression values independent of their composition, shape, or mass. Osmometers are devices used to measure the effects of solute concentrations on the thermodynamic properties of solutions. Essentially, osmotic measurements are based on changes in the free energy of solvent molecules that occur when a solute is dissolved in the solvent. Osmometers generally fall into three categories: 1) freezing point osmometers, 2) vapor pressure osmometers, and 3) membrane osmometers. The focus of the present invention concerns, particularly, freezing point osmometers.

There are two basic designs and procedures that are used in freezing point osmometry. First, there is the more conventional freezing point osmometer which generally operates to supercool a liquid sample below its freezing point with a spatially uniform temperature distribution. One such device is available from Wescor (Logan, Utah). The solution is then agitated (nucleated) which causes the solution to freeze. Upon freezing, the sample releases heat (as the heat of fusion) and causes the sample to rise in temperature to thermodynamic equilibrium at the freezing point. The freezing point can be observed as a plateau in the temperature of the sample before the sample cools again. The temperature is detected and read with thermocouples.

The second type of freezing point osmometer is generally based on the principles described by Ramsey and Brown in "Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid." *J. Sci. Instrum.* **32**:372 (1955). The general principle involves observing a solution sample with a magnifying device, lowering the temperature of the sample uniformly in space, in a controlled manner, and measuring the temperature of the sample with a measuring device, such as a thermocouple. The sample is first frozen, then remelted, partially, util a few ice crystals are left. The temperature of the sample is then dropped again, uniformly in space. The sample is continuously monitored with the magnifying device and the observations correlated to the temperature measurements. The temperature at which the sample is observed to start freezing (i.e., where the remaining ice crystals begin to grow) is taken to be the freezing point. A commercial version of a device using this principle is the Clifton osmometer which employs small microliter wells in a copper plate to hold the sample. Another scientific version is described in DeVries et al. "Freezing resistance in some antarctic fishes." *Science* **163**:1074 (1969).

The work that led to the development of the TGO of the present invention was motivated by our research relating to a new class of proteins, known as antifreeze proteins (AFP) or thermal hysteresis proteins (THP). It has been discovered that certain species of fish, insects, and plants when exposed to low or subfreezing temperatures, produce extracellular proteinaceous macromolecules with unique properties that do not follow classical osmometric properties. Ananthanarayanan "Antifreeze proteins: structural diversity and mechanism of action." *Life Chem. Rep.* 7:1-33 (1989); DeVries et al. *supra*; Raymond et al. "Adsorption inhibition as a mechanism of freezing resistance in polar fishes." *Proc. Natl. Acad. Sci. U.S.A.* **74**:2589 (1977); Duman et al. in *Water and Life* pp. 282-300 (Somero et al. eds. Springer-Verlag, New York/Berlin (1992)); Knight et al. "Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role." *Cryobiology* **23**:256-263 (1986); Urrutia et al. "Plant thermal hysteresis proteins." *Biochem. Biophys.*

Acta **1121**:199-206 (1992). These proteins cause anomalous hyper-colligative (greater than the colligative) freezing temperature depression for their concentration.

While the freezing temperature depression caused by such proteins is hyper-colligative, the melting temperature is unaffected and colligative. Based on these properties, the proteins were named "antifreeze proteins" (AFP), to describe their possible physiological function or "thermal hysteresis proteins" (THP), to describe their physical effect on freezing and melting.

Researchers became interested in THPs because of their unique thermodynamic properties and their possible practical value in bestowing cold tolerance and freeze tolerance on animals and plants. New species of animals and plants that produce THPs in response to low temperature exposure are being identified continuously. Usually the proteins are identified by gathering biological fluids from plants or animals after low temperature exposure and measuring the fluid freezing and melting temperatures to find hyper-colligative freezing temperature depression.

The study and identification of THPs require the use of an osmometer to measure freezing and melting temperatures in the same sample, to determine thermal hysteresis. Because many organisms, such as insects, have limited quantities of biological fluids and the THPs are often purified in small quantities, the osmometer must be able to measure phase transition temperatures of small volume samples.

The most common methods for measuring the freezing temperature depression and thermal hysteresis of THP solutions use direct microscopic observation of the sample as it is cooled and warmed on a spatially uniform time-dependent temperature stage in the presence of an ice-crystal seed (i.e., the Clifton osmometer). The temperature at which the observed seed begins to grow or shrink determines the freezing and melting points, respectively. A major problem with these types of optical osmometers, as with any osmometer that operates by measuring temperature, is their resolution.

Osmometers typically measure temperature with thermocouples or thermistors which have, at best, a resolution of 0.02°C. OMEGA "The Temperature Handbook," p. Z-25 (Stamford, CT (1992)). Consequently, the lowest limit of THP activity that can be measured is about 30 milliosmols (mOsm). This is determined through use of the relationship of the depression of the freezing temperature depression and osmolality of $\Delta T \approx 1.86 \bullet C$, where C is the solute concentration in osmols and ΔT is the freezing temperature depression in °C. However, THPs occur in nature in low millimolar concentrations with colligative freezing temperature depression of 10^{-3}-10^{-4}°C. Most of the known THPs are not miscible in water at higher concentrations. Therefore, the THPs anomalous freezing temperature depression can be detected with available osmometers only because the depression is several orders of magnitude greater than the colligative freezing temperature depression. However, smaller anomalies in freezing temperature depression or colligative melting temperatures cannot be detected in THPs with existing devices.

It will be appreciated that freezing and melting point osmometry is fraught with limitations which complicate the technique and render it undesirable for many applications. First, accuracy of the devices is limited by the accuracy of the thermocouples employed. Indeed, the nature of heat transfer problems is that they are inherently boundary value problems and unless one can specify the boundary, one cannot specify the precise heat transfer relationship or the error. Thus, thermocouple inaccuracies can lead to unknown imprecisions in measurement of osmometric relationships. At best, the resolutions of the devices are limited by the resolution of the temperature measurement apparatus. In addition to thermocouple imprecision, most commercial freezing point osmometers (with the exception of the Clifton-type osmometer) require relatively large sample sizes. Large sample sizes are not practical for many biological and experimental measurements. Further, Clifton-type osmometers rely for their determination of the freezing and melting temperature on a relatively subjective visual determination by an operator of the onset of crystal growth processes.

Temperature gradients have been utilized to cause phase transformation in samples. For example, a procedure by Bridgman has been used to grow crystals across a temperature gradient. See Fleming "Solidification Processes" (McGraw Hill (1974)). Temperature gradients have also been employed by the present inventor in several contexts. For example, in U.S. Patent No. 4,531,373, a directional freezing device for the controlled freezing of biological samples was disclosed. The device generally included a first base and a second base which could be maintained at independent temperatures. The bases were separated by a gap and were connected by a substrate spanning the gap. Through holding the first and second bases at a first and second temperature (T_H and T_C), respectively, the substrate would experience a temperature gradient T_H - T_C. Samples could be moved across the substrate and cooled across the gradient. The apparatus and method allowed highly controlled freezing of biological samples. In another application, the inventor proposed a method to perform studies on the solid-liquid interface and ice crystal formation using a similar apparatus to that just described. Rubinsky et al. "Experimental Observations and Theoretical Studies on Solidification Processes In Saline Solutions". *Experimental Thermal & Fluid Science* **6(2)**:157 (1993).

A similar method was used by Fisher who developed a device that employs a temperature gradient and in which the temperature of the freezing interface is measured with an extremely fine thermocouple set underneath the location of the change of the phase interface. See Fisher "*Aspect of faceted and nonfaceted eutectic growth studied by means of organic analogues*" D.Sc. Thesis No. 301, E'cole Polytechnique Federale de Lusanne (1978).

As will be appreciated, each of the above systems utilized thermocouples to detect and measure temperatures of freezing and/or melting. As mentioned, thermocouples are not highly accurate. Moreover, the systems required relatively large sample volumes. Accordingly, a need exists in the art for a freezing point osmometer that has enhanced resolution and accuracy. Further, it would be advantageous to provide an osmometer that required small sample

sizes. In particular, it would be desirable to have a device meeting these objectives for the study of antifreeze proteins.

SUMMARY OF THE INVENTION

In accordance with the present invention there is provided a freezing point osmometer that is capable of high precision and degrees of accuracy. Moreover, in preferred embodiments, the osmometer of the invention utilizes very small sample sizes (i.e., on the order of microliters or nanoliters of sample or below). The invention includes both apparatus for achieving these objectives as well as methods for performing osmometry.

In general, apparatus in accordance with the present invention includes a substrate generally separated into three zones: a first zone, a second zone, and a gradient zone. The gradient zone generally extends between the first and second zone. The first and second zone are adapted to be independently varied in temperature such that a temperature gradient can be established across the gradient zone. The gradient zone includes a plurality of sample rows or channels extending generally from the first zone to the second zone.

In a preferred embodiment, the osmometer further comprises a first temperature controller in communication with the first zone and a second temperature controller in communication with the second zone, the first and second temperature controller being adapted to independently vary the temperature of the first and second zone. In another preferred embodiment, the osmometer further comprises a detector for detecting a solid-liquid interface in the sample rows. In another preferred embodiment, the detector comprises an imaging device. In another preferred embodiment, the detector comprises a magnifying device. In another preferred embodiment, the magnifying device comprises a microscope. In another preferred embodiment, the osmometer further comprises a video camera in optical communication with the magnifying device. In another preferred embodiment, the osmometer further comprises a computer for capturing a video image from the magnifying device. In another preferred embodiment the osmometer further comprises at least three sample

rows, comprising a first, a second, and a third sample row, wherein, the first and second sample rows comprise standard solutions having a first and a second known phase transition temperature and the third sample row includes a solution whose phase transition temperature is desired to be quantified. In another preferred embodiment, the first phase transition temperature is higher than the phase transition temperature of the unknown solution and the second phase transition temperature is lower than the phase transition temperature of the unknown solution. In another preferred embodiment, the first zone further comprises a first base and the second zone further comprises a second base, the first and second bases being separated by the gradient zone and each of the first and second bases being in heat transfer relation with the substrate. In another preferred embodiment, the sample rows comprise capillary tubes.

In accordance with a second aspect of the present invention, there is provided a thermal-gradient osmometer, comprising a substrate having a first end, a second end, a first surface, and a second surface, the second surface having a plurality of sample rows extending generally from the first surface to the second surface, a first base having a first heat transfer surface adapted to sit in heat transfer relation with the first surface of the substrate, a first temperature controller in communication with the first base for controlling a temperature, T_H, of the first base to be above the phase transition temperature of samples contained in the sample rows, a second base spaced a distance, d, from the first base to define a gap therebetween and having a second heat transfer surface adapted to sit in heat transfer relation with the first surface of the substrate, and a second temperature controller in communication with the second base for controlling a temperature, T_C, of the second base to be below the phase transition temperature of samples contained in the sample rows.

In a preferred embodiment, the osmometer further comprises a substrate mover for moving the substrate generally longitudinally across the first base in the direction of the second base with the first surface in heat transfer relation with both of the first heat transfer surface and the second heat transfer surface. In another preferred embodiment, the temperature, T_H, is controlled to be

substantially constant, the temperature, T_C, is controlled to be substantially constant, and the substrate mover moves the substrate at a substantially constant velocity. In another preferred embodiment, the temperatures, T_H and T_C, are so chosen and the substrate mover moves the substrate at a velocity chosen such that the samples in the sample rows undergo phase transformation when opposite the gap. In another preferred embodiment, the osmometer further comprises a magnifying device positioned opposite the gap and being adapted for viewing solid-liquid interfaces in the sample rows. In another preferred embodiment, the magnifying device comprises a microscope. In another preferred embodiment, the osmometer further comprises an imaging device in optical communication with the magnifying device. In another preferred embodiment, the osmometer further comprises a computer for capturing a video image from the imaging device.

In accordance with a third aspect of the present invention, there is provided a method to perform freezing point osmometry, comprising providing in a first sample row a first solution having a first phase transition temperature, wherein the first freezing point is desired to be quantified, providing in a second sample row a second solution of a second known phase transition temperature, the second phase transition temperature being different than that of the first solution, providing in a third sample row a third solution of a third known phase transition temperature, the third phase transition temperature being different than that of the first and second solution, exposing the first, second, and third sample rows longitudinally across a substantially linear temperature gradient that extends from a first temperature that is higher than the phase transition temperature of the first solution to a second temperature that is lower than the phase transition temperature of the third solution so as to cause a portion of the first, second, and third solutions to undergo phase transformation and to generate respective first, second, and third solid-liquid interfaces at a respective first, second, and third positions in the sample rows, detecting the respective first, second, and third solid-liquid interfaces in the sample rows and quantifying the first phase

transition temperature from the position of the first solid-liquid interface in relation to the positions of the second and third solid-liquid interfaces.

In acordance with a fourth aspect of the present invention, there is provided a method to perform osmometry, comprising: providing a substrate having a first zone, a second zone, and a gradient zone, the first and second zone being adapted to be independently varied in temperature such that a temperature gradient may be established across the gradient zone, the gradient zone having at least three sample rows extending generally from the first zone to the second zone, each of the sample rows being adapted to be filled with a sample, providing in a first sample row a first standard solution having a known first phase transition temperature that is different than that of an unknown sample whose phase transition temperature is to be determined, providing in a second sample row a second standard solution having a known second phase transition temperature that is different than that of the unknown sample and the second sample, providing in a third sample row the unknown sample, creating a temperature gradient across the gradient zone, the temperature gradient extending from a first temperature that is higher than the first phase transition temperature to a second temperature that is lower than the second phase transition temperature, allowing the substrate and the sample rows to come to thermodynamic equilibrium and to cause a portion of the first, second, and unknown samples to undergo phase transformation within the sample rows and create a first, second, and third solid-liquid interface within the sample rows, and detecting the relative positions of the first, second, and third solid-liquid interfaces and determining the phase transition temperature of the unknown sample at the third solid-liquid interface from the known first and second phase transition temperatures at the first and second solid-liquid interfaces.

In preferred embodiments of each of the third and fourth aspects of the invention, the sample rows comprise capillary tubes. In another preferred embodiment, the sample rows are contained within a substrate. In another preferred embodiment, the method further comprises the steps of: raising the second temperature so as to cause a portion of the first, second, and third

solutions to melt and to generate respective fourth, fifth, and sixth solid-liquid interfaces at a respective fourth, fifth, and sixth positions in the sample rows, detecting the respective fourth, fifth, and sixth solid-liquid interfaces in the sample rows and quantitating the first phase transition temperature from the position of the fourth solid-liquid interface in relation to the positions of the fifth and sixth solid-liquid interfaces. In another preferred embodiment, the sample rows comprise capillary tubes. In another preferred embodiment, the sample rows are contained within a substrate.

In accordance with a fifth aspect of the present invention, there is provided a method to screen for molecules having antifreeze (AF) or thermal hysteresis (TH) activity, comprising: providing a thermal-gradient osmometer, comprising a substrate separated into a first zone, a second zone, and a gradient zone, the gradient zone generally extending between the first and the second zone, the first and the second zone being adapted to be independently varied in temperature such that a temperature gradient can be established across the gradient zone, wherein the gradient zone includes a plurality of sample rows extending generally from the first zone to the second zone, providing in a first sample row a first standard solution having a known first phase transition temperature that is different than that of an unknown sample which is to be screened for AF or TH activity, providing in a second sample row a second standard solution having a known second phase transition temperature that is different than that of the unknown sample and the second sample, providing in a third sample row the unknown sample, creating a temperature gradient across across the gradient zone, the temperature gradient extending from a first temperature that is higher than the first phase transition temperature to a second temperature that is lower than the second phase transition temperature, allowing the substrate and the sample rows to come to thermodynamic equilibrium and to cause a potion of the first, second, and unknown samples to undergo a first phase transformation within the sample rows and create a first, second, and third solid-liquid interface within the sample rows, and detecting the relative positions of the first, second, and third solid-liquid interfaces, raising the second temperature so

as to cause a portion of the first, second, and unknown solutions to undergo a second phase transformation and to generate respective fourth, fifth, and sixth solid-liquid interfaces at a respective fourth, fifth, and sixth positions in the sample rows, detecting the respective fourth, fifth, and sixth solid-liquid interfaces in the sample rows, and determining the phase transition temperature of the unknown sample at the third solid-liquid interface from the known first and second phase transition temperatures at the first and second solid-liquid interfaces, and determining the phase transition temperature of the unknown sample at the sixth solid-liquid interface from the known fourth and fifth phase transition temperatures at the fourth and fifth solid-liquid interfaces,

wherein, an unknown sample possesses AF or TH activity when there is a difference in the phase transition temperature of the unknown sample between the first phase transformation and the second phase transformation.

As will be appreciated, the temperature can be changed through either physically raising and/or lowering the temperature or through physically moving the samples or the substrate relative to the temperature gradient.

In accordance with a sixth aspect of the present invention, there is provided a molecule possessing antifreeze or thermal hysteresis activity identified according to the screening process.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

FIGURE 1 is a schematic representation of the temperature gradient osmometer of the present invention showing a transverse view of the device. The figure shows two independently variable temperature zones marked T_H and T_C separated by a gap with a substrate (i.e., a microslide) extending therebetween which creates a temperature gradient T_H-T_C across the substrate. Samples in the sample rows (i.e., capillary tubes) rest on the substrate. The optical image is recorded by a video camera and is displayed to a monitor. A top view of the substrate and four sample rows is shown in Figure 1a where the solid-liquid freezing interface can be seen separating the frozen (dark) lower region from the unfrozen (light) upper region.

FIGURE 2 is a top perspective view of an alternative design of the substrate which, in a single, unitary piece includes a first zone, a second zone, and a gradient zone.

FIGURE 3 is a top perspective view of a substrate having *in situ* sample rows.

FIGURE 4 is another top perspective view of a substrate having *in situ* sample rows.

FIGURE 4A is another top perspective view of a substrate having *in situ* sample rows, wherein the sample rows are discontinuous.

FIGURE 5 is a laser printed picture of a captured image of a sample run on the TGO using light polarized microscopy. Five capillary tubes containing (labelled from left to right (a) to (e)) a distilled water sample (HPLC quality, 1 ppm residue) (tube (a)), followed by a 4.25 mM aqueous NaCl solution (tube (b)), a 8.5 mM aqueous NaCl solution (tube (c)), a 12.75 mM aqueous NaCl solution (tube (d)), and a 17 mM aqueous NaCl solution (tube (e)) are shown with respective solid-liquid interfaces. The freezing interfaces appear as horizontal lines inside the capillary tube, separating the frozen lower region from the unfrozen upper region.

FIGURE 6 is a laser printed picture of a captured image of a sample run on the TGO using light polarized microscopy. Five capillary tubes containing (labelled from left to right (a) to (e)) distilled water samples (HPLC quality, 1 ppm residue) (tubes (a) and (e), 1.7 mM Alanine in water (tube (b)), 0.17 mM Alanine in water (tube (c)), and 0.017 mM Alanine in water (tube (d)) are shown with respective solid-liquid interfaces. The freezing interfaces appear as horizontal lines inside the capillary tube, separating the frozen lower region from the unfrozen upper region.

FIGURE 7 is a laser printed picture of a captured image of samples run on the TGO using light polarized microscopy. Seven capillary tubes containing (labelled from left to right (a) to (e)) tube (a) = water; tube (b) = 137 mM aqueous NaCl; tube (c) = THP I from the Northern winter flounder, *Pleuronectes americanus*, average molecular weight (MW) 3.6 kDa, (A/F

Protein, Boston); tube (d) = THP II from the sea raven, *Hemitriphase transitionerus americanus*, average MW 14 kDa (A/F Protein, Boston); tube (e) = THP III from the Newfoundland ocean pout, *Macrozoarces americanus*, average MW 6.7 kDa, (A/F Protein, Boston); tube (f) = AFGP 1-8 from Antarctic notothenid fish, *Dissostichus Mawsoni*, average MW 4 kDa (DeVries, A.L., University of Illinois, Urbana Champaign); and tube (g) = water are shown with respective solid-liquid interfaces. The freezing interfaces appear as horizontal lines inside the capillary tube, separating the frozen lower region from the unfrozen upper region.

FIGURE 8 is a laser printed picture of a captured image of samples run on the TGO using light polarized microscopy. The samples are the same as those depicted in Figure 7, but are shown during melting. To effect melting, the temperature gradient was changed, i.e., the T_H and T_C were set to different values, which alters the optical resolution through changing the distances separating the solid-liquid interfaces.

FIGURE 9 is a laser printed picture of a captured image of samples run on the TGO using light polarized microscopy. Six capillary tubes containing, as labelled from left to right tubes (a) through (f), water (HPLC quality, 1 ppm residue) (tube (a)), 0.625 mM aqueous poly-D-Lys (6.7 kDa) (tube (b)), 1.25 mM aqueous poly-D-Lys (6.7 kDa) (tube (c)), 2.5 mM aqueous poly-D-Lys (6.7 kDa) (tube (d)), 5 mM aqueous poly-D-Lys (6.7 kDa) (tube (e)), and 1 mM aqueous AFGP 1-8 (DeVries, University of Illinois at Urbana-Champaign) (tube (f)) are shown with respective solid-liquid interfaces. The freezing interfaces appear as horizontal lines inside the capillary tube, separating the frozen lower region from the unfrozen upper region.

FIGURE 10 is a laser printed picture of a captured image of samples run on the TGO using light polarized microscopy. The samples shown in Figure 9 are shown during melting as described in Figure 8.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Introduction

We have developed a new freezing point osmometer that operates on a temperature gradient. The osmometer of the invention is referred to herein as a temperature-gradient osmometer (TGO). The TGO of the invention can perform with extremely small sample volumes, can measure both freezing and melting temperatures in a single sample, and has a resolution many orders of magnitude greater than that of existing osmometers. Using this device we found anomalies in the depression of the freezing temperature and thermal hysteresis in aqueous solutions of hydrophilic amino acids, poly-amino acids and lectins. These anomalies would not have been possible to detect with currently used technology. Hydrophilic amino acids, poly-amino acids and lectins have been reported in the literature as having the ability to bind to cell membranes. In view of our discovery that such molecules also possess thermal hysteresis and hyper-colligative freezing temperature depression, a relationship between a molecule's ability to bind to cell membranes and its anomalous freezing temperature depression is indicated.

With our prototype we measured phase transition temperatures with a resolution of 10^{-5}°C, and we estimate that the resolution of the device can be increased to 10^{-8}°C with commercially available technology. In comparison, freezing point osmometers of the prior art record resolutions of 10^{-2}°C to 10^{-3}°C. Using the TGO of the present invention we identified several organic molecules that possess hyper-colligative freezing temperature depression and thermal hysteresis. These results are important because they show that these properties may exist in many organic molecules. THPs are special because they have very high hyper-colligative freezing temperature depression, which facilitated their earlier identification with less accurate osmometers.

Relationship Between Thermodynamic Properties and Cell Membrane Binding

Our results together with evidence from literature, suggest that compounds which possess thermal hysteresis and hyper-colligative freezing temperature depression also have the ability to bind to cell membranes and therefore these appear to be related properties. For example, the following listing indicates certain correlations between thermal hysteresis and hyper-colligative temperature depression and cell membrane binding potential:

1.) It was recently determined that the different types of fish THPs which possess thermal hysteresis can also interact with membranes and protect mammalian cells and organs during hypothermic storage and cryopreservation. Hays et al. "Interaction of antifreeze glycoproteins with liposomes." *Biophys. J.* **64**:8296 (1993); Rubinsky et al. "The effect of antifreeze glycopeptides transition ides on membrane potential changes at hypothermic temperatures." *Biochem. Biophys. Res. Comm.* **173**:1369 (1990).

2.) Herein, we demonstrate that Threonine and Lysine have thermal hysteresis and hyper-colligative freezing temperature depression, albeit with smaller freezing temperature depressions than those of previously identified THPs. An earlier study showed that Threonine and Lysine can interact with plant cell membranes and contribute to freeze tolerance in plants. Finale et al. in *Cryopreservation of Plant Cells and Organs* pp. 75-113 (Kartha ed., CRC Press, Florida (1985)).

3.) We demonstrate herein that poly-Lysine has thermal hysteresis and specific colligative coefficients comparable to those of fish THPs. Poly-Lysine is also widely used as a strong cell adhesion protein. Jacobson et al. "Coupling polylysine to glass beads for plasma membrane isolation" *Biochimica et Biophysica Acta* **506(1)**:81-969 (1978).

4.) Finally, previous evidence suggests that lectins are homologous to THP II and can modify the ice crystal structure in a similar fashion as THPs. Ewart et al. "Structural and functional similarity between fish antifreeze proteins and calcium-dependent lectins."

Biochem. Biophys. Res. Comm. **185**:335 (1992); Urrutia *supra*. Our discovery herein that lectins also have thermal hysteresis and hyper-colligative freezing temperature depression is consistent with the correlation of such properties and cell membrane binding potential.

The observation that hyper-colligative freezing temperature depression and membrane binding ability may be related properties could resolve questions concerning plants and insects that produce THPs with small thermal hysteresis following exposure to cold. Duman *supra*; Urrutia *supra*. It appears that the low level of thermal hysteresis contributed by the proteins is not sufficient to contribute to freeze tolerant plant and insect survival and the reason for the proteins' appearance following low temperature exposure is not entirely clear. While it has been suggested that the THP's may function to inhibit recrystallization (Knight et al. "Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role." *Cryobiology* **23**:256-263 (1986)), the answer is not clear. The TGO of the present invention should assist in the elucidation of the problem.

If, in general, proteins which interact with cell membranes also depress freezing temperatures hyper-colligatively, then it is possible that the THP function is related to their interaction with cell membranes and the thermal hysteresis and hyper-colligative freezing temperature depression is a manifestation of this property. This suggestion is supported by the finding that, Threonine and Lysine, which interact with cell membranes to protect membranes from freeze injury, have, as described herein, hyper-colligative freezing temperature depressions and thermal hysteresis. Threonine and Lysine have not previously been identified as possessing thermal hysteresis, because the degree of thermal hysteresis was beyond the range of resolution of existing osmometers.

It should be emphasized, however, that irrespective of the purpose for which THPs may have first formed in animals and plants, their ability to induce thermal hysteresis may have found direct functions in such animals as the Antarctic an North Atlantic fish.

The possible correlation between an organic compound's interaction with cell membranes and modification of freezing temperatures appears reasonable. Without wishing to be bound to any particular theory or mode of operation, it is believed that THPs affect freezing temperatures by binding to ice crystals. Raymond et al. "Adsorption inhibition as a mechanism of freezing resistance in polar fishes." *Proc. Natl. Acad. Sci. U.S.A.* 74:2589 (1977). Cell membranes, like ice crystals, interact with the surrounding water. An organic molecule that binds with membranes will replace the water adjacent to the membrane. The binding process must be thermodynamically advantageous for these same molecules to bind to ice crystals while replacing the liquid water surrounding the ice.

The TGO of the present invention is uniquely adapted to carry out studies to further demonstrate the "antifreeze" activity of additional membrane binding proteins. Accordingly, the TGO of the invention provides a superb assay for the relation between a protein's ability to bind to ice crystals and to adhere to cell membranes leading to a new method to study adhesion protein function and structure and a better understanding of protein structure and the interaction between proteins, cell membranes, and water.

The Temperature-Gradient Osmometer of the Invention

Apparatus in accordance with the invention operate by establishing a space-temperature correlation in a domain. The space-temperature correlation can be established by introducing samples with a known phase transition temperature in the domain and recording the location of the phase transition interface of these samples. The phase transition temperature of a sample with an unknown composition can be determined from the known space-temperature correlation by introducing that sample in the domain, observing the spatial location of the phase transition interface and inferring (through interpolation or extrapolation) the temperature from the space-temperature correlation.

As used herein, a "solution" refers to a pure liquid compound, a mixture of pure liquid compounds, a pure liquid compound or mixture of pure liquid

compounds having a solute dissolved therein, a mixture of liquids having a solute dissolved therein, and the like. "Liquid," as used herein, can refer to solids which become liquids at a given temperature, i.e., metals, alloys, and the like.

Referring now to Figure 1, there is provided a schematic representation of an apparatus that can be used to implement the above general principles. The TGO of the invention includes an elongated substrate 20 spanning two independently variable temperature zones or bases 21 and 22 (labeled T_C and T_H respectively) separated by a gap 23. A plurality of sample rows 24 (Fig 1a; 24a-24d) are provided on or in the substrate 20. The sample rows 24 in a simple embodiment are square or rectangular capillary tubes. As will be appreciated, when the temperature in base 21 (T_C) and the temperature in base 22 (T_H) are set so that they are different, a temperature gradient T_C-T_H is established across the substrate 20 and in the sample rows 24 across the gap 23 separating base 21 from base 22.

Where the temperatures in base 21 and base 22 are selected so that the temperature gradient established crosses the phase transition temperature of samples provided on the substrate 20, a clear demarcation between the solid and liquid phase in the samples is visible. Such results are shown schematically in Figure 1a. There, the solid phase is shown in dark and the liquid phase is shown in light in the sample rows 24a-24d. The differences between the phase transition temperatures in sample rows 24a/24d and 24b and 24c are clearly visible.

Returning now to Figure 1, the bases 21 and 22 in a simple embodiment are prepared from copper blocks through which a row is drilled, serving as a coolant passage (not shown). For example, liquid nitrogen can be used as a coolant and passed through the bases 21 and 22. The temperatures of the bases 21 and 22 can be independently controlled through a temperature controller 25 which may be in communication with thermocouples or thermistors 28 and 29 through lines 26 and 27 which can be utilized for relative feedback on the temperatures in bases 21 and 22. The controller 25 is also in communication and control, through lines 30 and 31, of temperature controllers or heaters 21a and

22a for the bases 21 and 22. For example, where coolant passage is used to cool the bases 21 and 22, thin 80 Ω thermofoil heaters (Minco Products, Inc.) may be used as heaters 21a and 22a and placed on the top surface of the bases and sandwiched beneath a metal plate (i.e., a 0.16 cm thick copper plates may be placed on the top surface of the bases 21 and 22).

Using the controller 25, the bases 21 and 22 can be maintained at different constant or variable temperatures. The control system in a preferred embodiment works by connecting the two thermocouples 28 and 29 to two controllers (Fuji Electric, PYZ4) which in turn are connected to the thermofoil heaters 21a and 22a. This arrangement allows control of the temperature of both bases 21 and 22 by providing the required heating to compensate for the cooling from the coolant flow. Usually, one base 22 is set at a temperature (T_H), which is higher than the phase transition temperature of the given sample, and the other base 21 is set at a temperature T_C, which is lower than the phase transition temperature of such sample.

The bases 21 and 22 are, in a preferred embodiment, adapted to be placed on a microscope stage for optical examination of the sample rows 24. The substrate 20 may be either optically transparent or not. Where the substrate 20 is transparent, a transmitting illuminator may be used. Where the substrate 20 is not transparent, a reflecting illuminator can be used. In a preferred embodiment, the substrate 20 is transparent and the system is set on a microscope 32 (i.e., Zeiss Universal) with a transmitting illuminator and light polarization. The bases 21 and 22 are mounted on the horizontal stage of the microscope 32 separated by a gap 23. The bases 21 and 22 are preferably arranged in the same plane to within a few μm.

In a preferred embodiment, the substrate 20 is transparent and comprises a glass microslide (26 mm x 76 mm x 1 mm) (Objecttranger, Germany) which spans the bases 21 and 22 across the gap 23. Thermal contact between the substrate 20 and the bases 21 and 22 can be enhanced through a variety of known methods, such as clipping the substrate 20 to the bases 21 and 22. For example, brass clips can be used. The temperature of the substrate 20 will vary from T_H

to T_C across the gap 23 between the bases 21 and 22. The apparatus is designed to generate a one dimensional temperature distribution in the substrate 20 across the gap 23.

The gap 23 is preset to a predetermined distance (i.e., approximately 2.5 mm). The gap 23 between the bases 21 and 22 is generally centered around the focal line of the microscope 32. Thermocouples 28 and 29 are located in thermodynamic contact with each of the bases 21 and 22 on each side of the gap 23. In a preferred embodiment, 66.72 μm type T thermocouples (Omega, Stamford, CT) are used.

The sample rows 24 (24a-d; Fig.1a) in a preferred embodiment include a plurality of rectangular capillary tubes (0.1 mm x 0.1 mm x 40 mm) (Vitro Dynamics, Inc., NJ) which rest on the substrate 20 and are in good thermal contact with the substrate 20. For example, as with the substrate 20 to the bases 21 and 22, the sample rows 24 may be attached to the substrate with brass clips to enhance thermal contact. The top view of the substrate 20 (microslide) with the sample rows 24a-24d (capillaries) is shown schematically in Figure 1a. The samples to be tested are introduced into the sample rows 24. As will be appreciated, the system allows the simultaneous analysis of multiple samples, for example, six or more samples.

The sample rows 24 are chosen to be large enough to avoid surface tension effects on the phase transition temperature. The freezing point depression due to surface tension effects is equal to the product of the Gibbs-Thomson coefficient and the inverse of the radius of curvature. For a discussion of the effects of surface tension on phase transition, see, for example, Carey *Liquid vapor phase change phenomena* (Hemisphere Publ. Co., Washington (1992)); Kurtz et al. *Fundamentals of solidification* (Trans. Tech. Publ. Switzerland (1984)); Pruppacher et al. *Microphysics of Clouds and Precipitation* (D.Reidel Publ. Co. Dordrech:Holland and Boston:USA (1980)).

In order to reduce effects of air on the system during testing, the bases 21 and 22, the gap 23, the substrate 20, and the sample rows 24 are preferably confined within a housing (not shown) which has either a quiescent controlled

atmosphere or a partial vacuum. The system is designed in such a way that the sample rows 24 easily take the temperature of the substrate 20. Consequently, the temperatures of the substrate 20, the sample rows 24, and the samples within the sample rows 24 vary one-dimensionally in the horizontal direction, across the gap 23 from temperatures of the bases 22 and 21 (T_H to T_C, respectively).

Accordingly, the TGO apparatus provides a unique one-dimensional correlation between position on the substrate 20 and temperature. The substrate 20 carrying the sample rows 24 with the samples can be translated across the gap, horizontally either from the high temperature base 22, T_H, to the low temperature base, T_C, or in reverse, to freeze or melt the samples in the sample rows 24, respectively. Alternatively, the temperatures of the bases 21 and 22 can be raised or lowered to effect freezing or melting.

Where movement of the substrate is desired, the TGO apparatus may be equipped with a motorized substrate positioner (not shown) which can be used to move the substrate 20 with a controlled velocity V in either direction relative to the bases 21 and 22. Such movement of the substrate acts to change the temperature gradient and temperature acting on a given point (i.e., in the image area of the gap 23) of the substrate 20 and can be used to either melt or freeze the samples in the sample rows 24 depending on the direction of movement. Thus, through moving the substrate 20 in the colder direction (i.e., toward base 21 (T_C)), the samples will freeze. Conversely, through moving the substrate 20 in the warmer direction (i.e., toward base 22 (T_H)), the samples will thaw.

The image in the area of the gap 23, where the analyzed events occur, may be recorded through an image capturing device, such as the microscope 32 with a video camera 33 (for example, a CCD/RGB model DXC-151, Sony) attached to the microscope 32. The video image from the camera 33 may be recorded on video cassette with a video cassette recorder 34 and/or displayed through a monitor 35.

Optionally, video images may be captured with a computer (not shown). For example, a PC based video capture board (Aitech Int. Corp. Vimager Pro 256) can be used to capture still images at 640 x 480 pixel resolution. Image

processing software (such as NIH-Image v. 1.54) can be used for image processing analysis. The NIH Image v.1.54 software is a Macintosh based software package. However, image files, captured on PC based systems, may be transferred from such systems to Macintosh systems through networks, or the like. Graphical analysis can then be performed on the Macintosh utilizing the image processing software. Micrographs may be printed using a laser printer.

The method of the invention may be understood more particularly from Figure 1a. There, four sample rows 24a-24d are loaded with various samples. In a preferred embodiment, the sample rows 24a and 24d are loaded with a sample that is a standard which has a higher melting point than an unknown sample contained in sample row 24c. In sample row 24b, a second standard sample which has a lower melting point than the unknown sample contained in sample row 24c. The samples contained in sample rows 24a and 24d can be used to ensure that the gradient is one dimensional across the substrate 20, varying only linearly across the gap 23, and not laterally across the substrate 20. Thus, a baseline isotherm in a direction normal to that of the one-dimensional temperature variation can be established. These two exterior sample rows (24a and 24d) together with the interior sample row 24b with the other sample with a known composition are used to establish the space-temperature correlation. Therefore, the solid-liquid interface in the unknown sample contained in sample row 24c can be compared in distance to the solid-liquid interfaces of the known samples contained in sample rows 24d and 24b to determine the melting point of the unknown sample in sample row 24c with a high degree of accuracy.

The axial temperature distribution in the space bounded by the phase transition interfaces in the standards contained in sample rows 24 (i.e., 24a, 24b, and 24d) is determined from knowledge of each control solution's composition, and by assuming a one-dimensional, linear temperature distribution between the phase transition interfaces. The phase transition temperature of the sample with the unknown composition is determined by linear interpolation from measurements of the axial distance between the phase transition interface locations of the tested sample and the controls. The distances in the axial

direction between the phase transition interfaces in the various sample rows 24 can be measured with light microscopy resolution. The temperature measurement resolution can be continuously increased by replacing the control solutions with compositions more closely matching the phase transition temperature of the tested sample and by generating shallower temperature gradient across the gap 23.

A variety of standard solutions having known freezing and melting point temperatures to high degrees of accuracy are well known in the art. For example, the *[CRC Handbook of Critical Temperatures]* provides tables of solutions formulated with particular freezing points which can be used as standard solutions in accordance with the invention.

As was mentioned above, the substrate can be movable (i.e., with a slide mover) so that the sample rows can be moved across the temperature gradient between the bases 21 and 22. In such a case, the substrate 20 having the sample rows 24 (containing solutions for calibration and solutions with unknown phase transition temperatures) are loaded on the high temperature side of the gradient (i.e., on base 22). To measure freezing phase transition temperature the motorized carriage with the substrate 20 and the sample rows 24 is pushed from the base 22 to the base 21 (T_H to T_C). After freezing starts at the cold end of the sample rows 24, the motor is stopped and measurements of distances between the phase transition interfaces are taken once the system has reached thermal equilibrium. In general, care is taken to freeze less than 5% of the volume of the sample so as to minimize the change in the composition of the unfrozen solution due to the possible rejection of solutes by ice in the frozen solution. The phase transition temperature during melting is determined similarly by translating or moving the sample rows 24 on the substrate 20 in the reverse direction (i.e., T_C to T_H).

Alternatively, rather than moving the substrate, the temperature of the bases 21 and 22 can be initially established to be same (i.e., both at a temperature above the phase transition temperature of the samples in the sample rows 24). Over time, the temperature of a base (i.e., base 21) can be lowered slowly to effect freezing of the samples in the sample rows 24 as observed in the

image area of the gap 23. Phase transition temperatures during melting can be measured through simply raising the temperature of the base (i.e., base 21) until melting is observed in the image area of the gap 23.

As will be appreciated, the TGO system of the present invention can be manufactured in a variety of different manners. A variety of these additional embodiments are described below:

The Substrate

The substrate of the TGO of the present invention can be designed as a single piece including the temperature control features and the like. An exemplary embodiment of a single piece substrate is shown in Figure 2 which is a top perspective view of an alternative design of the substrate. In the embodiment, in general, apparatus in accordance with the present invention includes a substrate 40 generally separated into three zones: a first zone 41, a second zone 42, and a gradient zone 43. The gradient zone 43 generally extends between the first and second zone 41 and 42. The first and second zone 41 and 42 are adapted to be independently varied in temperature such that a temperature gradient can be established across the gradient zone 43. The gradient zone includes a plurality of sample rows 44 extending generally from the first zone 41 to the second zone 42. The first zone 41 is typically separated from the second zone 42 by a recess 45 under the gradient zone 43 which assists in generation of the temperature gradient across the gradient zone 43.

The substrate 40 can be formed of a single piece of material (as shown) or can be manufactured from a variety of materials. For example, in the embodiment pictured in Figure 1, the substrate is manufactured from several materials and components. However, the bases 21 and 22 with the substrate 20 (in Figure 1) operate equivalently to the first zone 41, the second zone 42, and the gradient zone 43 (in Figure 2). The first zone 41 and the second zone 42 are typically connected to a heating/cooling system and/or controller as is discussed in connection with Figure 1.

The Sample Rows

As will be appreciated, the sample rows utilized in the present invention can be as simple as capillary tubes (Figs. 1 and 1a) or can be etched or imbedded into the substrate. All that is generally required for a sample row to be effective in accordance with the invention is that the sample row extend generally longitudinally along the substrate in good thermal contact with the temperature gradient. The sample rows, therefore, are generally elongate but their particular geometry is not exceedingly important. One requirement of the sample rows is that a sample is exposed to a range of temperatures across the temperature gradient, whatever geometry of the sample row that is chosen.

Thus, for example, an etched or imbedded sample row design is provided in Figure 3 which is a top perspective view of a substrate having *in situ* sample rows. The sample rows 54 comprise rows within the substrate 50 extending generally from the first zone 51 to the second zone 52 across the gradient zone 53. At one end the sample rows 54 preferably include sample wells 56 which allow for easy loading of the sample rows 54 with sample. Samples may be pipetted into the sample wells 56 and will fill the sample rows 54 through capillary action. The sample rows 54 are preferably covered from the sample wells 56 over a portion of their length (i.e., extending over the gradient zone 53 of the substrate 50). This assists in limiting any air effects as well as creates very fine resolution of the solid-liquid interface upon freezing across a temperature gradient. Where the sample wells 56 are covered, to facilitate filling of the sample rows 54, the sample rows 54 may include at the opposite end from the sample wells 56, vent holes 57.

The substrate, as described above, can be manufactured from one or more components. For example, where temperature control of the gradient is accomplished utilizing bases (Fig. 1; 21 and 22), the substrate may be prepared as shown in Figure 4. Or, alternatively, the substrate can be manufactured as a single piece as shown in Figure 3. An advantageous construction is to manufacture the substrate as a single piece of glass including the temperature

control apparatus. The sample rows 54 in such embodiment can be etched or embedded using common techniques known in the art.

It will be appreciated that where sample rows are made (i.e., etched or embedded) directly in the substrate there is a greater ability to line up the samples to ensure consistency and uniformity. Moreover, greater heat transfer control is facilitated.

In addition, it is also possible to construct a device wherein the sample rows are discontinuous, i.e., a plurality of wells in a row. This embodiment is shown in Figure 4A.

Solid-Liquid Interface Detection

As described above in connection with Figure 1, the solid-liquid interface is detected optically. However, as will be appreciated, alternative methods to detect the solid-liquid interface are contemplated in accordance with the invention. Essentially, all that is necessary in accordance with the invention is to be able to detect the relative positions of the solid-liquid interfaces of the samples.

Therefore, any detection system which enables such detection will be useful in the present invention. For example, ultrasonic imaging techniques are highly effective at detecting interfaces between materials. In addition, there are other optical systems that operate on edge detection principles. Also, it may be possible to detect thermal differences between the solid phase and the liquid phase, such as differences in electrical conductivity. This later embodiment could be particularly useful in an embodiment where the sample rows are embedded or etched in the substrate, such as the embodiment described in connection with Figures 3 and 4.

With advances in solid state physics and chemistry, there are practically daily developments in liquid crystal technology. Certain liquid crystals can be designed to change color in response to changes in temperature. Accordingly, we expect that liquid crystal type designs might be incorporated into the TGO of

the present invention for aiding in edge detection or as replacements for sample rows containing standard solutions.

Through utilizing appropriate solid-liquid phase detection apparatus, automation of the TGO will become possible and user interaction can be limited to preparation of samples to be tested.

Mode of Operation and Examples

Experiments were done with the TGO to characterize the system and to investigate the phase transition temperatures of various aqueous solutions of interest. The operation of the TGO of the present invention will become apparent through the following discussion and examples in conjunction with the accompanying Figures.

Example 1.

In this example, we measured the freezing point of water in comparison to several sodium chloride solutions to view the freezing point depressions in the solutions and to gather insight into the resolution of the TGO of the present invention. An embodiment similar to that described in conjunction with Figure 1 was used.

The results are shown in Figures 5 which is a laser printed picture of a captured image of a sample run on the TGO. In Figure 5, five capillary tubes containing (labelled from left to right (a) to (e)) a distilled water sample (HPLC quality, 1 ppm residue) (tube (a)), followed by a 4.25 mM aqueous NaCl solution (tube (b)), a 8.5 mM aqueous NaCl solution (tube (c)), a 12.75 mM aqueous NaCl solution (tube (d)), and a 17 mM aqueous NaCl solution (tube (e)). The axes of the capillary tubes are in the Figure's vertical direction. All Figures discussed herein have similar orientation.

In Figure 5, the freezing interface in the samples appears as a convex line, separating the frozen lower region from the unfrozen upper region. A computer generated straight line was drawn through the midpoints of the freezing interfaces in the two exterior columns. The freezing point of the water sample (tube (a)) is known to be 0°C and the freezing point of the 12.75 mM aqueous NaCl solution (tube (e)) is known to be -0.062°C. The axial distance between the phase transition interfaces in the two exterior capillaries (tubes (a) and (e)), therefore, corresponds to a temperature difference of 0.062°C.

The phase transition temperatures in the interior samples (tubes (b) through (d)) are found by interpolation of axial distance from the phase transition interface of the exterior capillaries (tubes (a) and (e)). For example, a perpendicular line is drawn across the sample from the low temperature sample in tube (e). The distance is measured from the high temperature line in tube (a). Here, the distance is 2.5 cm. The distance is measured for each of the other samples: tube (b) = 1.8 cm; tube (c) = 1.2 cm; and tube (d) = 0.7 cm. These

are subtracted from the 2.5 cm total gradient distance for the 0.062°C depression to determine the linear degree of depression. This result is subjected to the ratio:

$$\frac{0.62}{2.5} = \frac{y}{x}$$

where y = the linear degree of depression in cm; and
x will equal the depression in degrees centrigrade.

These results are shown in the following Table:

TABLE 1

Tube	Distance	Temperature Depression
Tube (b)	.7	-0.0138
Tube (c)	1.3	-0.0322
Tube (c)	1.8	-0.0446

The linear distribution of the phase transition interfaces in the interior capillaries (tubes (b) through (d)) demonstrate the accuracy of the device. A temperature resolution of ± 0.003°C is therefore achieved in the experiment.

Example 2.

In this example, we measured the freezing point of water in comparison to several aqueous solutions of the amino acid Alanine (Spectrum, Gardena, CA) to view the freezing point depressions in the solutions and to gather insight into the resolution of the TGO of the present invention. An embodiment similar to that described in conjunction with Figure 1 was used.

The results are shown in Figure 6 which is a laser printed picture of a captured image of a sample run on the TGO. In Figure 6, five capillary tubes containing (labelled from left to right (a) to (e)) distilled water samples (HPLC quality, 1 ppm residue) (tubes (a) and (e), 1.7 mM Alanine in water (tube (b)), 0.17 mM Alanine in water (tube (c)), and 0.017 mM Alanine in water (tube (d)).

The freezing interface appears as a horizontal line inside the capillary tube, separating the frozen lower region from the unfrozen upper region. Figure 6 illustrates the degree to which the temperature distribution along the axes of the capillaries is one-dimensional. A computer generated straight line is drawn through the midpoints of the horizontal phase transition interfaces of the two exterior capillaries (tubes (a) and (e)) containing water.

The resulting line is not vertical to the axis of the capillary tubes because it is sometimes difficult to set the tubes in the precise direction of the temperature gradients. However, it should be noted that the line, which was computer generated by connecting the midpoints of the exterior freezing interfaces, passes through, and closely coincides with, both of the flat freezing interfaces in the exterior capillaries.

The computer generated line, defined by the freezing point interfaces in tube (a) to tube (e), defines the 0°C isotherm baseline. The distance of the phase transition interfaces in the interior capillaries (tubes (b) through (d)) can be measured from the 0°C baseline in a normal direction as described in Example 1. Thus, the temperature measurement accuracy is improved over what is possible in conventional freezing point osmometry. Figure 6 also illustrates the resolution of the TGO since the Alanine solution freezing temperature depression measured here cannot be measured with conventional osmometers.

Example 3.

In Examples 1 and 2, we noticed that the freezing point interface observed in the capillaries can be either straight as in Figure 5 or concave as in Figure 6. The shape of the line changes in the same sample with (i) the rate of freezing and melting that is used to achieve the equilibrium conditions and (ii) the temperature gradient. In particular, slower freezing and melting rates and shallower temperature gradients produce a straighter freezing interface line.

It is possible that the interface curvature may be caused by the difference in thermal conductivity between glass and ice. Therefore, regardless of the reason for the curvature, for standardization purposes, whenever the experiment

produces a curved surface we use the center of the ice column for measuring distances.

Example 4.

As was mentioned above, the TGO is particularly useful in the study of thermal hysteresis in thermal hysteresis protein (THP) solutions and other solutions. In this example, we examined and compared the freezing and melting temperatures of the four major types of fish THPs that have been characterized to date as described by Ananthananrayanan *supra*. The four proteins examined were THP I, THP II, and THI III and AFGP.

The results are shown in Figures 7 and 8, which are laser printed pictures of captured images of samples run on the TGO. In each of the tests, 1 mM aqueous solutions of the four fish THPs with a low temperature control solution of 137 mM NaCl and a high temperature control of pure water (HPLC quality, 1 ppm residue). The capillary tubes, in each of the Figures contained the following samples (labelled from left to right tubes (a) through (f)):

tube (a) water;

tube (b) 137 mM aqueous NaCl;

tube (c) THP I from the Northern winter flounder, *Pleuronectes americanus*, average molecular weight (MW) 3.6 kDa, (A/F Protein, Boston);

tube (d) THP II from the sea raven, *Hemitriphase transitionerus americanus*, average MW 14 kDa (A/F Protein, Boston);

tube (e) THP III from the Newfoundland ocean pout, *Macrozoarces americanus*, average MW 6.7 kDa, (A/F Protein, Boston);

tube (f) AFGP 1-8 from Antarctic notothenиid fish, *Dissostichus Mawsoni*, average MW 4 kDa (DeVries, A.L., University of Illinois, Urbana Champaign); and

tube (g) water.

The results in Figure 7 demonstrate that, consistent with earlier findings (Ananthananrayanan *supra*; DeVries *supra*), the freezing point temperature depression of the THP's is anomalous and hyper-colligative, about two orders of magnitude higher than the colligative value. The THP solutions appear to have different freezing temperature depressions. This result could be due to either the different effects of the THPs on the freezing temperature depression or to errors in the approximate estimates of the THPs average molecular weight.

Both of Figures 7 and 8 show the typical spicular ice crystal structure that forms in the presence of THPs. Figure 8 shows the phase transition interface after melting and demonstrates that, as anticipated, there is thermal hysteresis in THP solutions. That is, the phase transition melting temperature is much higher than the phase transition freezing temperature. It should be noted that a different temperature gradient was used in the freezing experiment shown in Figure 7 and the melting experiment shown in Figure 8. The different gradient occurred because, rather than move the substrate to change the sample location in the gradient, the temperature across the gradient was changed on only one side. This was accomplished through raising the temperature on the low temperature side of the gradient (i.e., in Figure 1 the temperature of base 21 was raised while the temperature of base 22 was kept constant). Therefore, the distance between the phase transition interface in the NaCl capillary (tube (b)) and in the pure water capillary (tubes (a) and (g)) changed.

Example 5.

In an effort to establish negative controls for use in studying THPs, we measured the freezing temperature depression in several amino acids and poly-amino acids. The results were surprising.

However, prior to discussing the results, we will introduce a variable that can be used with the TGO of the invention as a quantitative measure for analyzing freezing temperature depressions. The following formula is commonly used to correlate osmolality and solution molality in the form:

osmolality (determined from phase transition temperature) = $v\ n\ m$;

where v is the osmotic coefficient;

n is the dissociation coefficient; and

m is molality.

v is a solvent-dependent, experimentally determined coefficient which is a function of concentration and normally has values of less than one. Values of v and n for NaCl solutions are well established. Partanen et al. "Freezing point depression of dilute aqueous sodium chloride solutions." *Acta Chemica Scandinavica* **45**:172-176 (1991). Accordingly, we can utilize the "specific osmotic coefficient" v_o, which is defined as the ratio between the osmotic coefficient of a substance v and the osmotic coefficient of a NaCl solution with the same product ($n\ m$) and pressure. With limited exceptions a substance that produces hyper-colligative freezing temperature depression will have a specific osmotic coefficient greater than one.

In accordance with the above, we conducted repeated measurements of 17 mM concentrations of aqueous solutions of Phenylalanine (Phe (Sigma, St. Louis, MO)), Methionine (Met (Spectrum, Gardena, Ca)), Threonine (Thr (Spectrum, Gardena, Ca)), and Lysine (Lys (Spectrum. Gardena, Ca)). The specific osmotic coefficient of Phe, Met, Thr, and Lys were measured as:

Phe 0.86 ± 0.1;

Met 0.86 ± 0.03;

Thr 1.12 ± 0.02; and

Lys 1.52 ± 0.04.

The results for the hydrophobic Phe and Met are not surprising since the specific osmotic coefficient is normally less than one. However, the results for the hydrophilic amino acids tested are surprising because of their ability to produce anomalous hyper-colligative freezing temperature depression, a property that was thought to be unique to THPs. Ananthananrayanan *supra*; DeVries *supra*; Duman *supra*; Urrutia *supra*. In addition, measuring phase transition temperature during melting shows that there is no anomalous phase transition temperature depression in any of the above amino acid solutions. These results

demonstrate that certain amino acids possess properties that were previously believed to be unique to THPs, namely, thermal hysteresis.

Example 6.

In addition to amino acids, we investigated the freezing temperature depressions of aqueous solutions of two commercially available poly-amino acids, poly-Proline (Pro (Sigma, St. Louis, MO)) and poly-Lysine (Lys (Sigma, St. Louis, MO)), each of which have good solubility in water. We found no hyper-colligative freezing temperature depression or thermal hysteresis in poly-Pro. However, the poly-Lys behaved very differently.

The results are shown in Figures 9 and 10, which are laser printed pictures of captured images of samples run on the TGO. The capillary tubes in Figures 9 and 10 contain, as labelled from left to right tubes (a) through (f), water (HPLC quality, 1 ppm residue) (tube (a)), 0.625 mM aqueous poly-D-Lys (6.7 kDa) (tube (b)), 1.25 mM aqueous poly-D-Lys (6.7 kDa) (tube (c)), 2.5 mM aqueous poly-D-Lys (6.7 kDa) (tube (d)), 5 mM aqueous poly-D-Lys (6.7 kDa) (tube (e)), and 1 mM aqueous AFGP 1-8 (DeVries, University of Illinois at Urbana-Champaign) (tube (f)).

A comparison between the freezing temperature depression in poly-Lys and AFGP 1-8, as shown in Figure 9, clearly demonstrates that the poly-Lys possesses hyper-colligative freezing temperature depression. Although five times less than the AFGP 1-8, the poly-Lys freezing temperature depression magnitude is considerable. Additional experiments, in accordance with Example 6, showed that the specific osmotic coefficient for aqueous poly-Lys at 1 mM is 45 ± 5. This value is comparable to that of aqueous AFGP 7-8 which at 1 mM is 61 ± 10.

In Figure 10, the phase transition interface during melting of the samples is shown. Clearly, both poly-Lys and AFGP exhibit thermal hysteresis.

Example 7.

Recently it was found that C-type lectins are homologous to type II THP's. Ewart et al. "Structural and functional similarity between fish antifreeze proteins and calcium-dependent lectins." *Biochem. Biophys. Res. Comm.* **185**:335 (1992). It has also been observed that C-type lectins can modify the morphology of ice crystals similar to THP's. Rubinsky et al. "Ice-crystal growth and lectins." *Nature* **360**:115 (1992).

Therefore, we used the TGO to measure the phase transition temperature of aqueous solutions of lectins from *Dolichos biflorus* (Sigma, St. Louis, MO). Measurements in accordance with Example 6 show that at a concentration of 1 mM, the lectin has anomalous hyper-colligative freezing temperature depression with a specific osmotic coefficient of 25 $\pm$ 12 and thermal hysteresis.

Example 8.

In this example, we describe a method for screening for molecules having antifreeze or thermal hysteresis activity. Basically, there are two approaches with reference to Figure 1. In each, at least one sample and two "controls" are used. In the first screenining process phase transformation is accomplished through changing the temperature across the gradient.

1. raise temperature of zone 3 or lower temperature of zone 1 to cause freezing across the gradient.

2. measure the phase transition temperature of the sample from the controls to determine freezing temperature of the sample.

3. lower temperature of zone 3 or lower temperature of zone 1 to cause melting across the gradient.

4. measure the phase transition temperature of the sample from the controls to determine melting temperature of the sample.

5. the difference between the temperature in steps 2 and 4 is the thermal hysteresis.

In this second example, instead of changing the temperatures, the substrate is moved across the gradient:

1. push samples or substrate across gradient from right to left (where temperature is colder on the left than right) and stop.

2. measure the phase transition temperature of the sample from the controls to determine freezing temperature of the sample.

3. push samples or substrate across the gradient in the opposite direction and stop.

4. measure the phase transition temperature of the sample from the controls to determine melting temperature of the sample.

5. the difference between the temperature in steps 2 and 4 is the thermal hysteresis.

As will be appreciated, this screening process allows one to quickly screen for thermal hysteresis and, consequently, likely antifreeze activity. Compounds can be quickly screened and identified through this process.

INCORPORATION BY REFERENCE

The following papers and publications are incorporated by reference herein in their entirety:

1. Ananthananrayanan "Antifreeze proteins: structural diversity and mechanism of action." *Life Chem. Rep.* 7:1-33 (1989);

2. DeVries et al. "Freezing resistance in some antarctic fishes." *Science* **163**:1074 (1969);

3. Carey *Liquid vapor phase change phenomena* (Hemisphere Publ. Co., Washington (1992));

4. Duman et al. in *Water and Life* pp. 282-300 (Somero et al. eds. Springer-Verlag, New York/Berlin (1992));

5. Ewart et al. "Structural and functional similarity between fish antifreeze proteins and calcium-dependent lectins." *Biochem. Biophys. Res. Comm.* **185**:335 (1992);

6. Finale et al. in *Cryopreservation of Plant Cells anel Organs* pp. 75-113 (Kartha ed., CRC Press, Florida (1985));

7. Fisher "*Aspect of faceted and nonfaceted eutectic growth studied by means of organic analogues*" D.Sc. Thesis No. 301, E'cole Polytechnique Federale de Lusanne (1978)

8. Hays et al. "Interaction of antifreeze glycoproteins with liposomes." *Biophys. J.* **64**:8296 (1993);

9. Jacobson et al. "Coupling polylysine to glass beads for plasma membrane isolation" *Biochimica et Biophysica Acta* **506(l)**:8l-969 (1978);

10. Knight et al. "Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role." *Cryobiology* **23**:256-263 (1986);

11. Kurtz et al. *Fundamentals of solidification* (Trans. Tech. Publ. Switzerland (1984));

12. OMEGA "The Temperature Handbook," p. Z-25 (Stamford, CT (1992));

13. Osuga et al. "Cooperative function between antifreeze glycoproteins." *J. of Biological Chemistry* **253**:6669 (1978);

14. Partanen et al. "Freezing point depression of dilute aqueous sodium chloride solutions." *Acta Chemica Scandinavica* **45**:172-176 (1991);

15. Pruppacher et al. *Microphysics of Clouds and Precipitation* (D.Reidel Publ. Co. Dordrech:Holland and Boston:USA (1980));

16. Ramsey et al. "Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid." *J. Sci. Instrum.* **32**:372 (1955);

17. Raymond et al. "Adsorption inhibition as a mechanism of freezing resistance in polar fishes." *Proc. Natl. Acad. Sci. U.S.A.* **74**:2589 (1977);

18. Rubinsky et al. "Experimental Observations and Theoretical Studies on Solidification Processes In Saline Solutions". *Experimental Thermal & Fluid Science* **6(2)**:157 (1993).

19. Rubinsky et al. "Ice-crystal growth and lectins." *Nature* **360**:115 (1992);

20. Rubinsky et al. "The effect of antifreeze glycopeptides transition ides on membrane potential changes at hypothermic temperatures." *Biochem. Biophys. Res. Comm.* **173**:1369 (1990);

21. Rubinsky et al. "A cryomicroscope using directional solidification for controlled freezing of biological materials." *Cryobiology* **22**:5548 (1985);

22. Sweeney et al. Limitations of methods of osmometry: measuring the osmolality of biological fluids." *Am. J. Physiol. 264 Regulatory Integrative Comp. Physiol.* **33**:R469-R480 (1993);

23. Urrutia et al. "Plant thermal hysteresis proteins." *Biochem. Biophys. Acta* **1121**:_____ (1992); and

24. Weast *Handbook of chemistry and physics* (___ ed.) (CRC, Florida (1984)).

CLAIMS

WHAT WE CLAIM IS:

1. A thermal-gradient osmometer, comprising a substrate separated into a first zone, a second zone, and a gradient zone, the gradient zone generally extending between the first and the second zone, the first and the second zone being adapted to be independently varied in temperature such that a temperature gradient can be established across the gradient zone, wherein the gradient zone includes a plurality of sample rows extending generally from the first zone to the second zone.

2. The osmometer of Claim 1, further comprising a first temperature controller in communication with the first zone and a second temperature controller in communication with the second zone, the first and second temperature controller being adapted to independently vary the temperature of the first and second zone.

3. The osmometer of Claim 1, further comprising a detector for detecting a solid-liquid interface in the sample rows.

4. The osmometer of Claim 3, wherein the detector comprises an imaging device.

5. The osmometer of Claim 3, wherein the detector comprises a magnifying device.

6. The osmometer of Claim 5, wherein the magnifying device comprises a microscope.

7. The osmometer of Claim 5, further comprising a video camera in optical communication with the magnifying device.

8. The osmometer of Claim 7, further comprising a computer for capturing a video image from the magnifying device.

9. The osmometer of Claim 1, further comprising at least three sample rows, comprising a first, a second, and a third sample row, wherein, the first and second sample rows comprise standard solutions having a first and a second known phase transition temperature and the third sample row includes a solution whose phase transition temperature is desired to be quantitated.

10. The osmometer of Claim 9, wherein the first phase transition temperature is higher than the phase transition temperature of the unknown solution and the second phase transition temperature is lower than the phase transition temperature of the unknown solution.

11. The osmometer of Claim 1, wherein the first zone further comprises a first base and the second zone further comprises a second base, the first and second bases being separated by the gradient zone and each of the first and second bases being in heat transfer relation with the substrate.

12. The osmometer of Claim 1, wherein the sample rows comprise capillary tubes.

13. A thermal-gradient osmometer, comprising:

a substrate having a first end, a second end, a first surface, and a second surface, the second surface having a plurality of sample rows extending generally from the first surface to the second surface;

a first base having a first heat transfer surface adapted to sit in heat transfer relation with the first surface of the substrate;

a first temperature controller in communication with the first base for controlling a temperature, T_H, of the first base to be above the phase transition temperature of samples contained in the sample rows;

a second base spaced a distance, d, from the first base to define a gap therebetween and having a second heat transfer surface adapted to sit in heat transfer relation with the first surface of the substrate; and

a second temperature controller in communication with the second base for controlling a temperature, T_C, of the second base to be below the phase transition temperature of samples contained in the sample rows.

14. The osmometer of Claim 13, further comprising a substrate mover for moving the substrate generally longitudinally across the first base in the direction of the second base with the first surface in heat transfer relation with both of the first heat transfer surface and the second heat transfer surface.

15. The osmometer of Claim 13, wherein the temperature, T_H, is controlled to be substantially constant, the temperature, T_C, is controlled to be substantially constant, and the substrate mover moves the substrate at a substantially constant velocity.

16. The osmometer of Claim 13, wherein the temperatures, T_H and T_C, are so chosen and the substrate mover moves the substrate at a velocity chosen such that the samples in the sample rows undergo phase transformation when opposite the gap.

17. The osmometer of Claim 13, further comprising a magnifying device positioned opposite the gap and being adapted for viewing solid-liquid interfaces in the sample rows.

18. The osmometer of Claim 17, wherein the magnifying device comprises a microscope.

19. The osmometer of Claim 17, wherein the osmometer further comprises an imaging device in optical communication with the magnifying device.

20. The osmometer of Claim 19, further comprising a computer for capturing a video image from the imaging device.

21. A method to perform freezing point osmometry, comprising:

providing in a first sample row a first solution having a first phase transition temperature, wherein the first freezing point is desired to be quantified;

providing in a second sample row a second solution of a second known phase transition temperature, the second phase transition temperature being different than that of the first solution;

providing in a third sample row a third solution of a third known phase transition temperature, the third phase transition temperature being different than that of the first and second solution;

exposing the first, second, and third sample rows longitudinally across a substantially linear temperature gradient that extends from a first temperature that is higher than the phase transition temperature of the first

solution to a second temperature that is lower than the phase transition temperature of the third solution so as to cause a portion of the first, second, and third solutions to undergo phase transformation and to generate respective first, second, and third solid-liquid interfaces at a respective first, second, and third positions in the sample rows;

detecting the respective first, second, and third solid-liquid interfaces in the sample rows and quantifying the first phase transition temperature from the position of the first solid-liquid interface in relation to the positions of the second and third solid-liquid interfaces.

22. The method of Claim 21, wherein the sample rows comprise capillary tubes.

23. The method of Claim 21, wherein the sample rows are contained within a substrate.

24. The method of Claim 21, further comprising the steps of:

raising the second temperature so as to cause a portion of the first, second, and third solutions to melt and to generate respective fourth, fifth, and sixth solid-liquid interfaces at a respective fourth, fifth, and sixth positions in the sample rows;

detecting the respective fourth, fifth, and sixth solid-liquid interfaces in the sample rows and quantitating the first phase transition temperature from the position of the fourth solid-liquid interface in relation to the positions of the fifth and sixth solid-liquid interfaces.

25. The method of Claim 24, wherein the sample rows comprise capillary tubes.

26. The method of Claim 24, wherein the sample rows are contained within a substrate.

27. A method to perform osmometry, comprising:

providing a substrate having a first zone, a second zone, and a gradient zone, the first and second zone being adapted to be independently varied in temperature such that a temperature gradient may be established

across the gradient zone, the gradient zone having at least three sample rows extending generally from the first zone to the second zone, each of the sample rows being adapted to be filled with a sample;

providing in a first sample row a first standard solution having a known first phase transition temperature that is different than that of an unknown sample whose phase transition temperature is to be determined;

providing in a second sample row a second standard solution having a known second phase transition temperature that is different than that of the unknown sample and the second sample;

providing in a third sample row the unknown sample;

creating a temperature gradient across the gradient zone, the temperature gradient extending from a first temperature that is higher than the first phase transition temperature to a second temperature that is lower than the second phase transition temperature;

allowing the substrate and the sample rows to come to thermodynamic equilibrium and to cause a portion of the first, second, and unknown samples to undergo phase transformation within the sample rows and create a first, second, and third solid-liquid interface within the sample rows; and

detecting the relative positions of the first, second, and third solid-liquid interfaces and determining the phase transition temperature of the unknown sample at the third solid-liquid interface from the known first and second phase transition temperatures at the first and second solid-liquid interfaces.

28. The method of Claim 27, wherein the sample rows comprise capillary tubes.

29. The method of Claim 27, wherein the sample rows are contained within a substrate.

30. The method of Claim 27, further comprising the steps of:

raising the second temperature so as to cause a portion of the first, second, and unknown solutions to melt and to generate respective fourth,

fifth, and sixth solid-liquid interfaces at a respective fourth, fifth, and sixth positions in the sample rows;

detecting the respective fourth, fifth, and sixth solid-liquid interfaces in the sample rows and quantitating the first phase transition temperature from the position of the fourth solid-liquid interface in relation to the positions of the fifth and sixth solid-liquid interfaces.

31. The method of Claim 30, wherein the sample rows comprise capillary tubes.

32. The method of Claim 30, wherein the sample rows are contained within a substrate.

33. A method to screen for molecules having antifreeze (AF) or thermal hysteresis (TH) activity, comprising:

providing a thermal-gradient osmometer, comprising a substrate separated into a first zone, a second zone, and a gradient zone, the gradient zone generally extending between the first and the second zone, the first and the second zone being adapted to be independently varied in temperature such that a temperature gradient can be established across the gradient zone, wherein the gradient zone includes a plurality of sample rows extending generally from the first zone to the second zone;

providing in a first sample row a first standard solution having a known first phase transition temperature that is different than that of an unknown sample which is to be screened for AF or TH activity;

providing in a second sample row a second standard solution having a known second phase transition temperature that is different than that of the unknown sample and the second sample;

providing in a third sample row the unknown sample;

creating a temperature gradient across across the gradient zone, the temperature gradient extending from a first temperature that is higher than the first phase transition temperature to a second temperature that is lower than the second phase transition temperature;

allowing the substrate and the sample rows to come to thermodynamic equilibrium and to cause a potion of the first, second, and unknown samples to undergo a first phase transformation within the sample rows and create a first, second, and third solid-liquid interface within the sample rows; and

detecting the relative positions of the first, second, and third solid-liquid interfaces;

raising the second temperature so as to cause a portion of the first, second, and unknown solutions to undergo a second phase transformation and to generate respective fourth, fifth, and sixth solid-liquid interfaces at a respective fourth, fifth, and sixth positions in the sample rows;

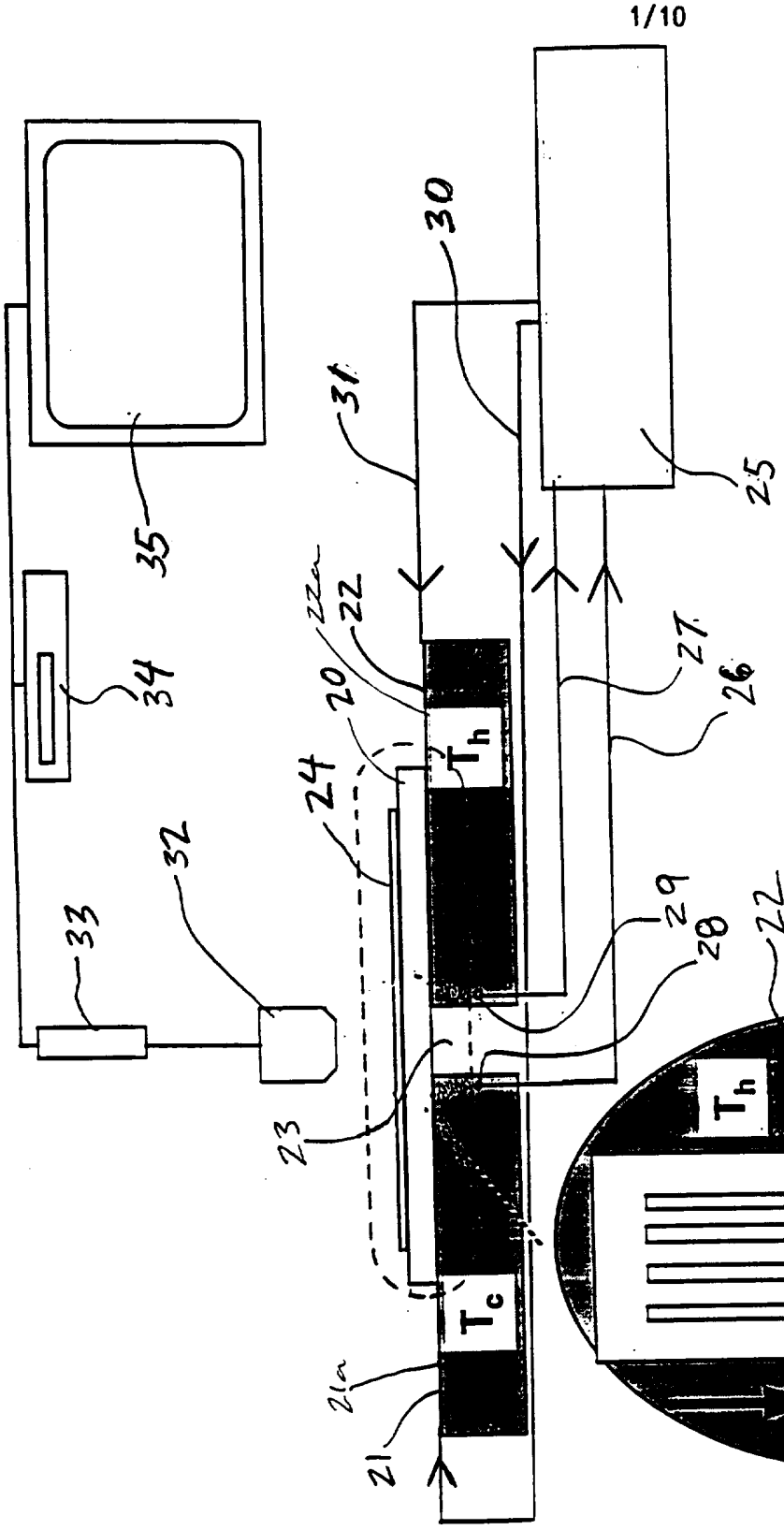
detecting the respective fourth, fifth, and sixth solid-liquid interfaces in the sample rows; and

determining the phase transition temperature of the unknown sample at the third solid-liquid interface from the known first and second phase transition temperatures at the first and second solid-liquid interfaces; and

determining the phase transition temperature of the unknown sample at the sixth solid-liquid interface from the known fourth and fifth phase transition temperatures at the fourth and fifth solid-liquid interfaces,

wherein, an unknown sample possesses AF or TH activity when there is a difference in the phase transition temperature of the unknown sample between the first phase transformation and the second phase transformation.

34. A molecule possessing antifreeze or thermal hysteresis activity identified according to the process of Claim 33.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31
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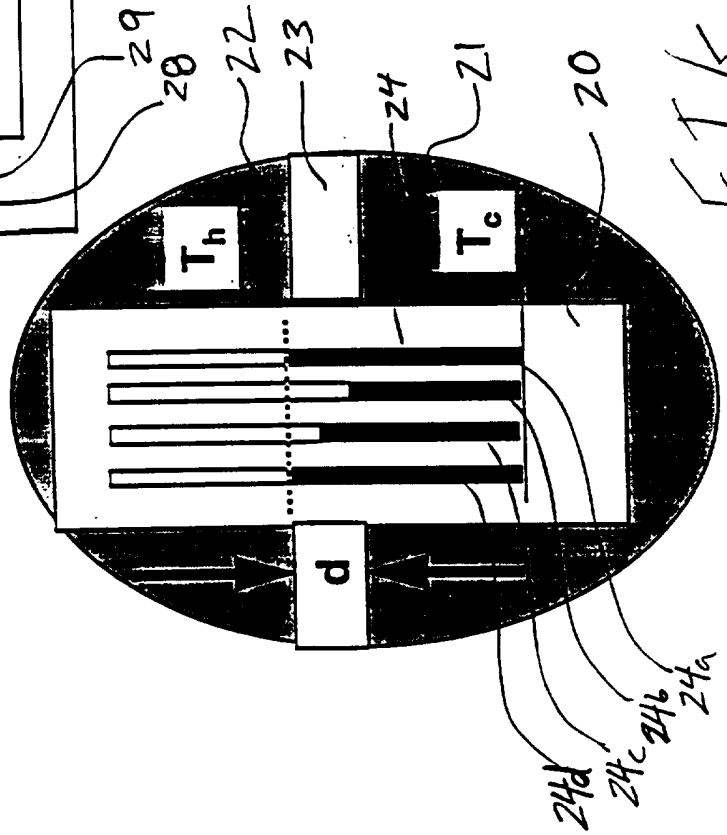
21a
21
T_h
T_c

FIG. 1


22
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T_h
T_c
d
24d
24c
24b
24a

FIG. 1a

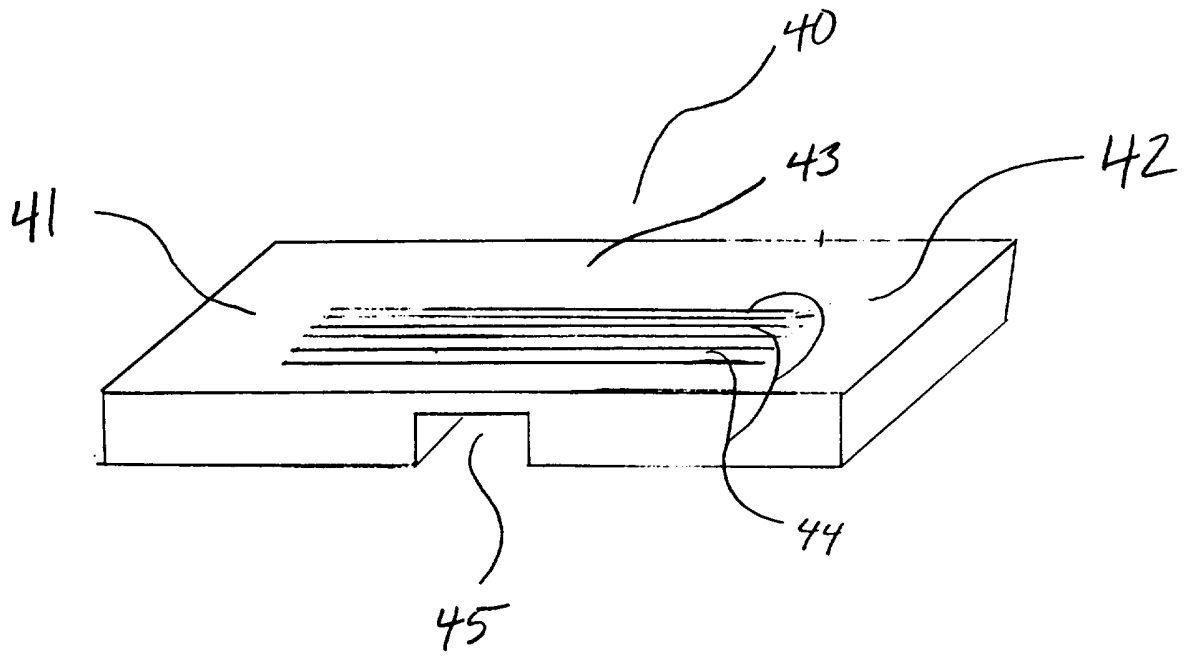

FIG. 2

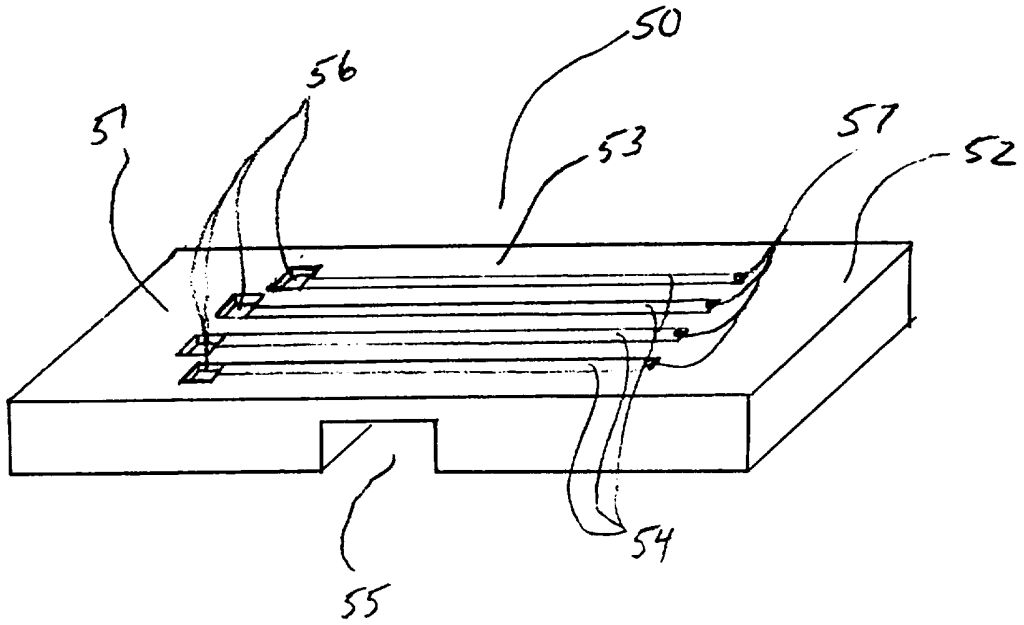

FIG. 3

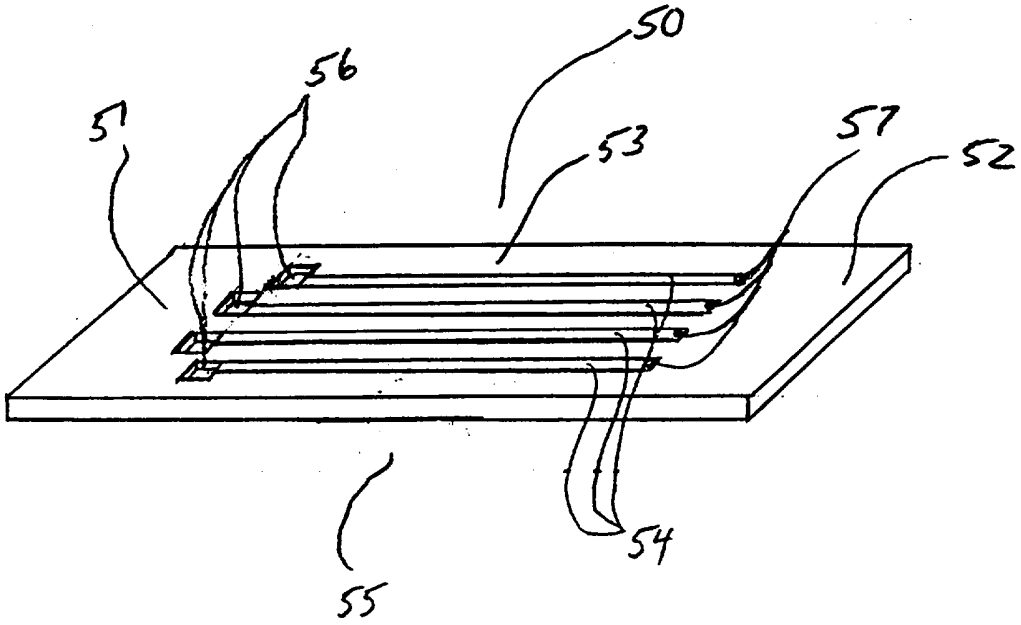

FIG. 4

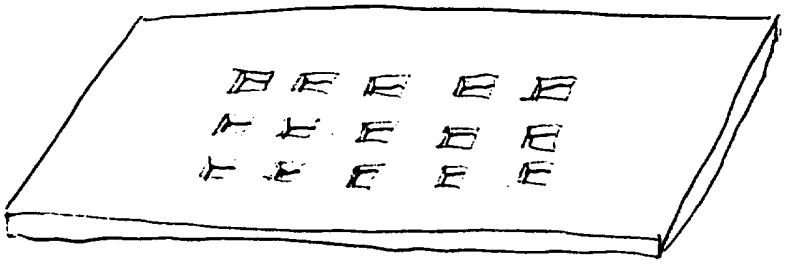

Fig. 4a

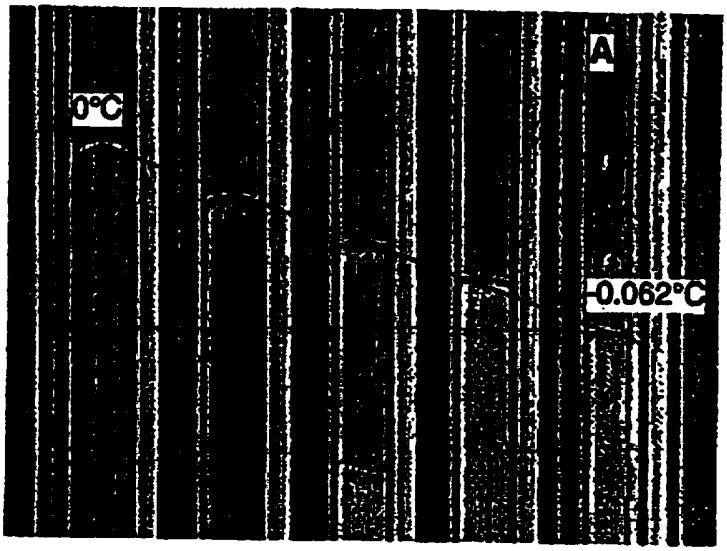

Fig. 5

-Fig 2a-

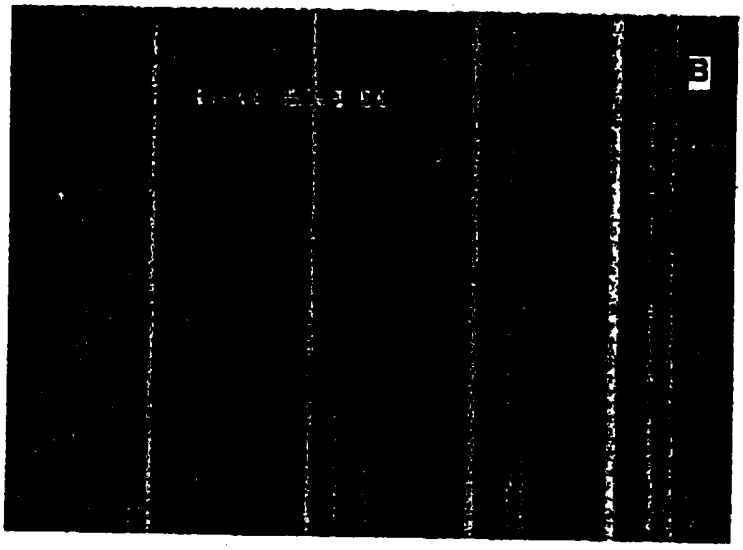

Fig. 6

- Fig25 -

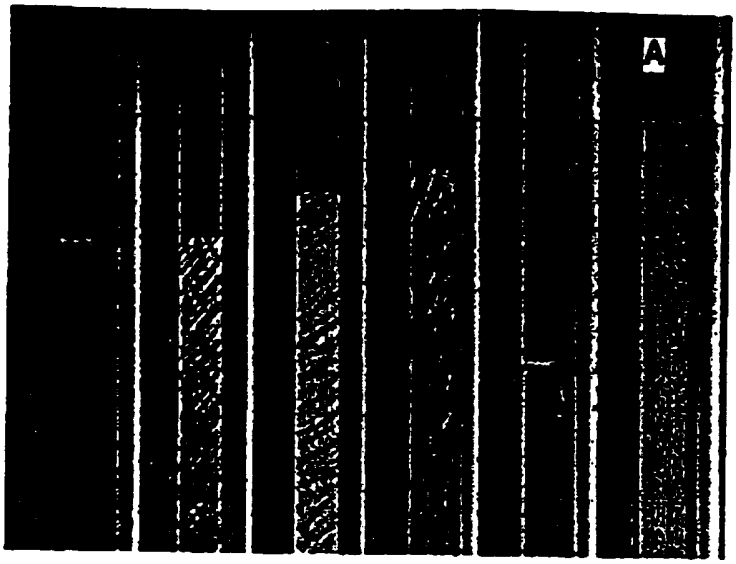

Fig. 7

– Fig 3a –

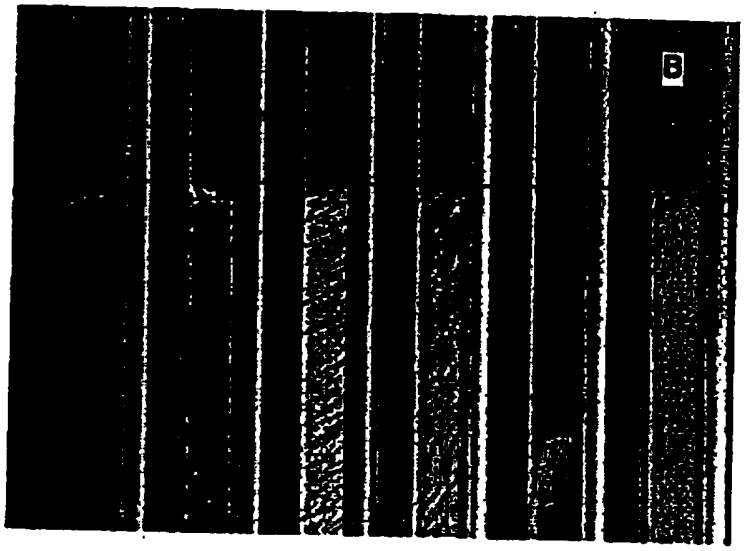

Fig. 8

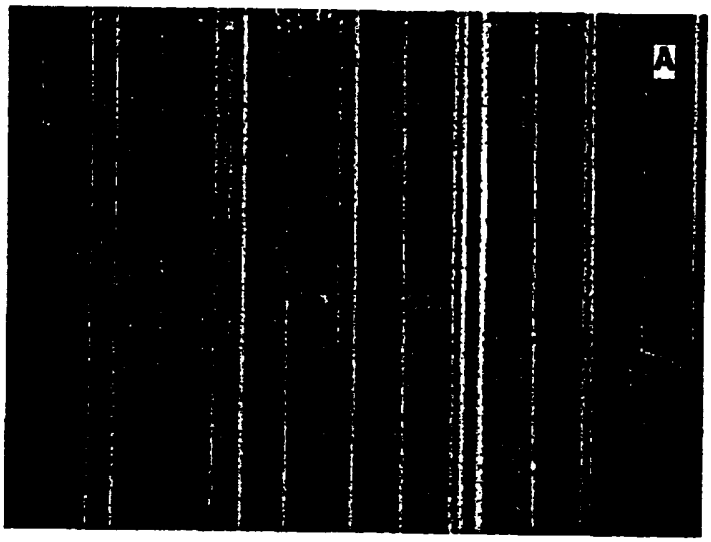

Fig. 9

- Fig 9a -

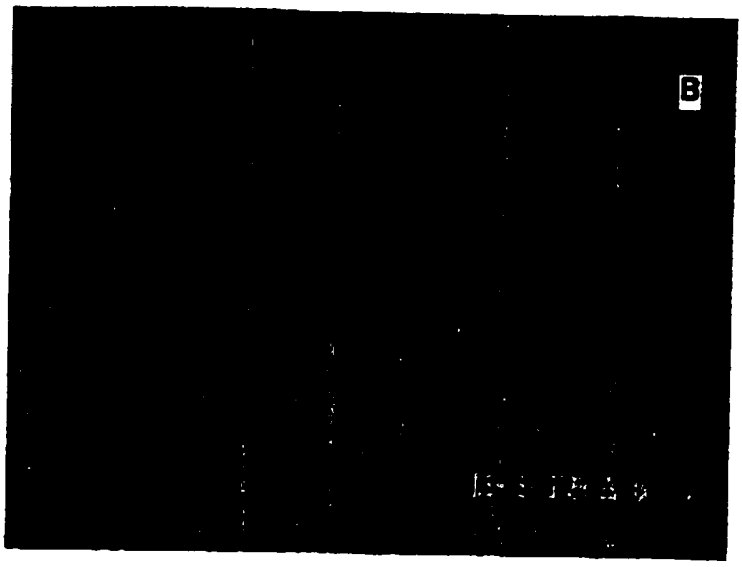

Fig. 10

- Figs -

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14352

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 25/04, 25/06; C07K 1/00
US CL :374/15,16,25,45; 530/305,350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 374/15,16,25,45,160; 530/305,350; 62/63,65

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,278,284 (LUSK ET AL.) 11 JANUARY 1994. SEE ENTIRE DOCUMENT, PARTICULARLY COLUMN 2, LINE 55, AND CLAIM 2.	34
Y	US, A, 2,691,885 (FARNHAM, JR.) 19 OCTOBER 1954. SEE ENTIRE DOCUMENT.	1-8, 11-13, 17-20
Y	JA, A, KOKAI NO. 52-23380 (MARUYAMA ET AL.) 22 FEBRUARY 1977. SEE ENTIRE DOCUMENT.	1, 3-8, 12-13, 17-20
A	US, A, 4,138,889 (FRASCHINI) 13 FEBRUARY 1979.	
A	US, A, 4,400,096 (MOLLOY) 23 AUGUST 1983.	
A	US, A, 2,730,892 (BRUCE ET AL.) 17 JANUARY 1956.	

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 JANUARY 1996	21 FEB 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DIEGO F.F. GUTIERREZ Telephone No. (703) 308-1113

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14352

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,383,770 (BOSCHUNG ET AL.) 17 MAY 1983.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14352

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14352

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

1. This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

a) Group I, claim(s) 1-32, drawn to an osmometer and to a method for performing freezing point osmometry.

b) Group II, claim(s) 33-34, drawn to a method for screening for molecules having antifreeze or thermal hysteresis activity and to a molecule having antifreeze or thermal hysteresis activity.

2. The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Inventions I and II are distinct and independent from each other because each invention is directed to a completely different method. In this case, Invention I is directed to an apparatus and method for determining a freezing point (phase transition temperature) while Invention II is directed to a method for screening molecules having antifreeze or thermal hysteresis activity which requires particular features or limitations not required in the other invention such as the step of determining if the sample has antifreeze or thermal hysteresis activity. Furthermore, the apparatus of Group I has separate utility since it can be used to practice another and materially different method such as a method for determining the dew point of a gas.