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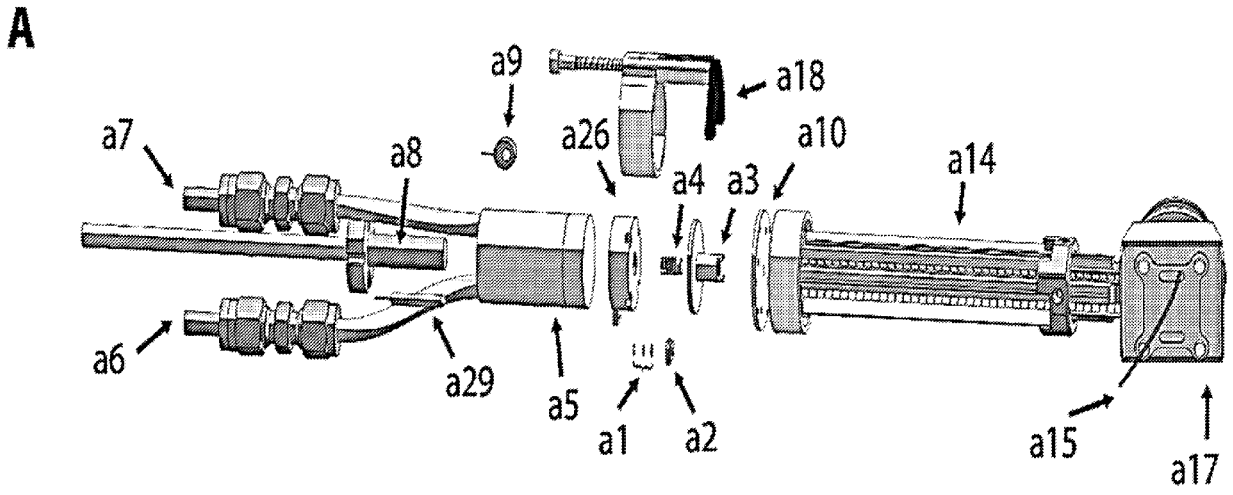


FIG. 1

(57) Abstract: Mass spectrometry technologies have been used in combination with cryo-electron microscopy (cryoEM) to obtain complete structural characterization. Fully integrating mass spectrometry with cryoEM would greatly improve sample preparation and accelerate the structural analysis. To that end, the present invention examines the use of soft-landing mass spectrometry to produce improved samples for cryoEM under vacuum or low pressures, including partially reheating samples to rehydrate landed particles to improve resolution of the cryoEM images.

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# CRYOGENIC SOFT LANDING IMPROVES STRUCTURAL PRESERVATION OF PROTEIN COMPLEXES

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims the benefit of priority to U.S. Provisional Patent Application Nos. 63/514,727, filed July 20, 2023, and 63/558,329, filed February 27, 2024, both of which is hereby incorporated by reference in their entirety.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

**[0002]** This invention was made with government support under GM118110 awarded by the National Institutes of Health. The government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

**[0003]** Single particle cryo-electron microscopy (cryo-EM) has become a leading technology for the determination of protein structure at high resolution, with even near-atomic and atomic resolution being possible (Cheng, Y., Science 2018, 361(6405): 876-880). A major bottleneck limiting the achievable resolution is the process by which protein–protein complex particles are suspended in thin films of amorphous ice, i.e., plunge freezing. With this technique—largely unchanged since its introduction forty years ago—particles often preferentially partition into the air–water interface, causing distortion upon freezing and inhibiting the random orientation that is required to solve the three-dimensional (3D) structure (Cianfrocco et al., Journal of Chemical Information and Modeling 2020, 60(5): 2458-2469). These issues, along with the difficulty of achieving a uniform desired ice thickness, make sample preparation a substantial obstacle and mandate considerable screening to identify particles with suitable orientations in the desired thickness of ice.

**[0004]** Recognizing these limitations, the possibility of using native mass spectrometry for cryo-EM sample preparation has been investigated as an alternative to plunge freezing. Native mass spectrometry involves the analysis and characterization of intact macromolecules, including but not limited to proteins and protein complexes, where the native structural features of the molecules are retained as much as possible.

**[0005]** Native mass spectrometry, which can determine molecule weight, sub-unit stoichiometry, and collisional cross sections, is already widely utilized as a complementary structural biology tool (Karch et al., Annual Review of Biophysics 2022, 51(1): 157-179). Furthermore, mass spectrometry (MS) requires relatively small amounts of material and has the flexibility to purify target molecules from complex mixtures. Deposition of the ion beam of

a MS onto a graphene or amorphous carbon transmission electron microscopy (TEM) grid can in principle offer a means to bypass the above limitations of conventional cryo-EM sample preparation while affording all the orthogonal benefits of MS.

**[0006]** Ideally, the mass spectrometer would generate cryo-EM samples by depositing the desired number of particles onto a grid in random orientations, while still providing a means to coat the particles with a thin film of amorphous ice. The amorphous ice is important for the downstream cryo-EM measurement as it provides some protection against environmental damage, such as radiation damage (Brown et al., Communications Biology 2022, 5(1): 817). However, there are many processes and steps involved with using MS devices to generate cryo-EM samples where the particles can suffer structural degradation. First, the protein complexes or other macromolecules must be ionized, vaporized, and subjected to the numerous DC and RF fields of the mass spectrometer. Second, the resulting particles must be gently deposited onto the surface of the TEM grid (typically comprising carbon or other suitable materials) with sufficient energy to adsorb but not so much as to cause structural damage. Third, given that deposition times typically range from one to 10 minutes, particles are exposed to a high vacuum environment for long durations, where presumably any solvent that was retained will quickly be lost.

**[0007]** Despite these challenges, considerable progress towards the goal of coupling MS with cryo-EM has recently been made (see, for example, U.S. Patent Nos. 11,092,523, 11,525,760, and 11,728,146, U.S. Publication No. 2023/0266214, and WO 2022/155306). Similar experiments were performed and, using negative-stain TEM, it was found upon imaging that particles deposited onto carbon grids at room temperature lacked the structural quality to enable 3D reconstructions (Lee et al., Journal of Proteome Research 2023, 22(3): 851-856; Salome et al., Analytical Chemistry 2022, 94(50): 17616-17624; and Westphall et al., Nature Communications 2022, 13(1): 2276).

**[0008]** This problem was solved by using a chemical landing matrix that preserves the deposited particles so that their 3D structures could be solved to the resolution of negative stain TEM (~ 30 A). In fact, the structures of the landed particles were virtually identical to those prepared conventionally. These matrix-landing experiments provided the best evidence to date that mass analyzed particles can retain their solution-phase structures. Unfortunately, the glycerol or polymeric matrices that were described are not directly compatible with cryo-EM. Others have constructed a device for the deposition of gas-phase cations of protein complexes onto TEM grids at room temperature followed by direct analysis with cryo-EM (Esser et al., Faraday Discussions 2022, 240: 67-80; and Fremdling et al., Acs Nano 2022, 16(9): 14443-14455). While the imaged particles lacked internal structure, the

overall shapes were correct and assays demonstrated that the landed particles still retained biological function.

**[0009]** Although the 3D reconstructions created using MS-based soft landing (alone or in combination with matrix materials) generally have the expected molecular shapes, resolution is limited by particle heterogeneity. To date, it has been extremely difficult to attain atomic resolution structures with cryo-EM with MS-based soft landing. One potential cause for this heterogeneity is that the landed ions are not as fully hydrated as they are in solution. Variability in the amount of internal and shell water molecules interacting with each particle causes differences in molecular structure from particle to particle, making convergence on the same final structure less likely. One possible solution to variability in particle hydration is to limit water loss in the vacuum of the mass spectrometer. However, this has proven challenging. As a result, alternative solutions are needed to improve structural heterogeneity and resolution.

**[0010]** Accordingly, what is needed are an apparatus and method able to deposit particles, including but not limited to proteins and protein complexes, onto surfaces compatible with cryo-EM analysis using MS devices, wherein the particles are able to retain their 3D structure while improving resolution.

## SUMMARY OF THE INVENTION

**[0011]** The present invention provides improved techniques and systems for cryogenically fixing analyte particles, such as proteins and other biological samples, in amorphous ice and other frozen amorphous solids, particularly for analysis by cryo-electron microscopy (cryo-EM). One aspect of the invention provides a landing apparatus and method that allows for temperature control of a receiving surface (including but not limited to TEM grids) within a mass spectrometer environment, sample (particle) deposition, formation of amorphous ice, warming of the sample (TEM grid) for rehydration of the particle, cooling for re-formation of amorphous ice, plunge freezing, and cryo-EM imaging.

**[0012]** In an embodiment of the present invention, samples are prepared by cooling analyte particles to very low temperatures prior to depositing the particles onto a cooled receiving surface, such as a TEM grid. The analyte particles are contacted with a vapor containing atoms or molecules preferably at cryogenic temperatures or near cryogenic temperatures (i.e., a temperature of -90°C or less, -150°C or less, -180°C or less, -190°C or less), and preferably under a vacuum or low pressure. As used in embodiments described herein, "under a vacuum" or "low pressure" refers to a pressure of $10^{-4}$ Torr or less, a pressure $10^{-5}$ Torr or less, or a pressure $10^{-6}$ Torr or less.

**[0013]** The temperature and pressure is such that the vapor is able to form a solid, preferably an amorphous solid, on, under, and/or around particles being deposited on the receiving surface. The analyte particles may be contacted by the vapor prior to the analyte particles being deposited on the receiving surface, concurrently with the analyte particles being deposited on the receiving surface, after the analyte particles are deposited on the receiving surface, or combinations thereof. As a result, a thin layer of a solid, preferably an amorphous solid, is formed around each particle, over each particle, under each particle, or combinations thereof.

**[0014]** In an embodiment, it is not necessary for the initial solid deposited on the receiving surface to be amorphous or completely amorphous. The heating techniques and associated cooling methods described herein can be used to convert crystalline solids (e.g., crystalline ice) to amorphous solids (e.g., amorphous ice). As a result, the techniques and methods described herein have the potential to simplify the process (sample preparation) of landing particles on the receiving surface. In addition, the heating and rehydration steps could also vitrify the solid into an amorphous solid that is suitable for electron microscopy.

**[0015]** The analyte particles may be charged ions, uncharged particles, or combinations thereof. In an embodiment, the analyte particles are ions generated from a mass spectrometer device, preferably after MS analysis has been performed on the particles. Suitable method for generating ions from particles include, but are not limited to, electrospray ionization and laser desorption, such as matrix-assisted laser desorption/ionization (MALDI).

**[0016]** Amorphous solids, or non-crystalline solids, refer to solids that lack the long-range molecular order characteristic of crystals. For example, the amorphous solids formed using the methods and systems described herein is preferably amorphous ice (also referred to herein as vitreous ice). Common $H_2O$ ice is a hexagonal crystalline material where the molecules are regularly arranged in a hexagonal lattice. In contrast, amorphous ice lacks the regularly ordered molecular arrangement. Amorphous ice and the other amorphous solids available with the present invention are generally produced either by rapid cooling of the liquid phase (so the molecules do not have enough time to form a crystal lattice) or by compressing ordinary ice (or ordinary solid forms) at very low temperatures.

**[0017]** In an embodiment, the present invention provides a cryo-sample preparation system comprising: a) a sample vacuum chamber; b) a sample holder positioned within the sample vacuum chamber, where the sample holder comprises a receiving surface; c) a first temperature control means able to provide a first temperature to the receiving surface of the

sample holder, where the first temperature is -20°C or less; d) an ion or particle generator comprising a vacuum housing having an internal pressure, where the ion or particle generator is able to produce a controllable analyte beam containing charged or uncharged analyte particles; e) a vapor source able to provide vapor to the sample vacuum chamber; and f) a second temperature control means able to provide a second temperature to the receiving surface of the sample holder, where the second temperature is greater than the first temperature.

**[0018]** The first and second temperature control means may be the same or different from one another. The vapor in the sample vacuum chamber is able to generate a solid around, under, and/or over the analyte particles deposited on the receiving surface. When the second temperature is applied to the receiving surface of the sample holder by the second temperature control means, a portion of the solid around, under, and/or over the analyte particles is converted to a liquid. The converted liquid is able to contact the deposited particles. Preferably, the solid is an amorphous solid. In an embodiment, the vapor comprises water vapor and the solid is an ice layer (preferably amorphous ice), which forms liquid water and interacts with the analyte particles to form a hydrated or rehydrated sample. In an embodiment, after the particles have been contacted with the converted liquid (or hydrated or rehydrated in the case of water), the converted liquid is cooled (preferably by the first temperature control means) to form or re-form an amorphous solid around the rehydrated sample, under the rehydrated sample, over the rehydrated sample, or combinations thereof.

**[0019]** The ion or particle generator is able to direct the analyte beam to contact the receiving surface of the sample holder, thereby depositing analyte particles onto the receiving surface, and the vapor in the sample vacuum chamber is able to generate a solid, preferably an amorphous solid, around, under, and/or over the analyte particles deposited on the receiving surface.

**[0020]** In an embodiment, the vacuum housing comprises a vacuum gate able to isolate the sample vacuum chamber from the ion or particle generator, so that the sample vacuum chamber is able to produce an internal pressure different from the internal pressure of the ion or particle generator. For example, when the vacuum gate is opened in order to direct the analyte beam to the sample holder, the sample vacuum chamber may have a slightly lower internal pressure than the ion or particle generator. Additionally, the vacuum gate is able to close and maintain the internal pressure of the ion or particle generator when the sample holder is removed from sample vacuum chamber.

**[0021]** Optionally, the ion or particle generator comprises a mass analyzer, including but not limited to time-of-flight (ToF), quadrupole, magnetic sector, ion trap, quadrupole ion trap,Orbitrap mass analyzers, or Astral analyzers, or combinations thereof, able to measure mass-to-charge ratios of ionized analyte particles, filter ionized analyte particles based on the mass-to-charge ratios of the particles, or combinations thereof.

**[0022]** In an embodiment, the ion or particle generator comprises an exit lens or aperture (such as an HCD cell exit lens or other ion optic exit lens) within the vacuum housing, where the exit lens or aperture is separated from the sample vacuum chamber by the vacuum gate. When the vacuum gate is opened, the ion or particle generator is able to direct the analyte beam though the exit lens or aperture into the sample vacuum chamber and to the receiving surface of the sample holder. Optionally, the system comprises an actuator able to move the sample holder toward the exit lens or aperture when the analyte beam is to contact the receiving surface, and move the sample holder away from the exit lens or aperture to a retrieval opening when it is time to extract the sample holder.

**[0023]** Optionally, the temperature control means is able to cool the analyte particles prior to the analyte particles being inserted into the sample vacuum chamber, being contacted by the vapor, or both. Alternatively, the ion or particle generator further comprises a separate temperature device able to cool the analyte particles prior to the analyte particles being inserted into the sample vacuum chamber, being contacted by the vapor, or both.

**[0024]** The vapor source is able to provide vapor to the sample vacuum chamber prior to the analyte particles being deposited on the receiving surface, concurrently with the analyte particles being deposited on the receiving surface, after the analyte particles are deposited on the receiving surface, or any combination thereof. The vapor may be added to the vacuum chamber as a molecular beam directed at the receiving surface (optionally provided by a doser, particularly a water doser), as part of the analyte beam containing the charged or uncharged analyte particles, as background gas present in the vacuum chamber as the analyte particles are deposited on the receiving surface, or combinations thereof.

**[0025]** In embodiments described herein, the vapor comprises any molecules or atoms able to form amorphous solids where exposed to low temperatures and pressures. Such vapors include, but are not limited to, cyclohexanol, methanol, ethanol, isopentane, water, $O_2$, Si, $SiO_2$, S, C, Ge, Fe, Co, Bi and mixtures thereof. Preferably, the vapor is water and the amorphous solid is amorphous ice. As with water, the amorphous state is obtained through condensation from the gas phase. Unlike water, which can be transformed to an amorphous solid by several techniques, non-water substance may require vapor-

condensation to form in the non-crystalline state (Zallen R., The Physics of Amorphous Solids, 1983, 8-10).

**[0026]** In an embodiment, the present invention provides a method for rehydrating an analyte on a substrate comprising the steps of: a) forming an analyte solution comprising analyte particles and a solvent; b) generating an analyte beam from the analyte solution, where the analyte beam comprises charged or uncharged analyte particles and molecules of the solvent; c) directing the analyte beam towards a receiving surface of a substrate under a first pressure such that the analyte particles and solvent molecules impinge on the receiving surface; d) forming a first solid layer of the solvent molecules on the receiving surface under a first temperature, where the first temperature is -20°C or less and the charged or uncharged analyte particles are embedded within the first solid layer; and e) heating the receiving surface to a second temperature, where the second temperature is greater than the first temperature. In a further embodiment, heating the receiving surface to the second temperature causes a portion of the first solid layer to undergo a phase change to a liquid solvent. A first portion of the liquid solvent interacts with the charged or uncharged analyte particles, and, in the case of water, forms a rehydrated sample.

**[0027]** In a further embodiment, the method for rehydrating the analyte further comprises forming an amorphous solid layer from a second portion of the liquid solvent under a third temperature of -20°C or less, where the rehydrated sample is embedded in the amorphous solid layer. As provided elsewhere in this description, the first temperature is -20°C or less, -40°C or less, -60°C or less, -90°C or less, -100°C or less, -130°C or less, -150°C or less, -175°C or less, -185°C or less, or -195°C or less; and the second temperature is -150°C or more, -140°C or more, -130°C or more, -120°C or more, -110°C or more, -100°C or more, -90°C or more, -80°C or more, -50°C or more, -20°C or more, -10°C or more, 0°C or more, or up to 5°C, with the provision that the second temperature is greater than the first temperature. The third temperature is -20°C or less, preferably -40°C or less, -60°C or less, -90°C or less, -100°C or less, -130°C or less, -150°C or less, -175°C or less, -185°C or less, or -195°C or less. In an embodiment, the first temperature is -150°C or less, the second temperature is -149°C or more, and the third temperature is -150°C or less.

**[0028]** During the deposition process, the internal pressure of the sample vacuum chamber is reduced to a pressure of $10^{-4}$ Torr or less, a pressure of $10^{-5}$ Torr or less, or a pressure $10^{-6}$ Torr or less. The ion or particle generator is operated at a pressure that is independent from the pressure of the sample vacuum chamber, typically a pressure of $10^{-3}$ Torr or less, a pressure of $10^{-4}$ Torr or less, a pressure of $10^{-5}$ Torr or less, or a pressure $10^{-6}$

Torr or less. To facilitate an increase in the sample holder temperature, the pressure of the sample vacuum chamber is optionally increased to 4.6 Torr or more, 10 Torr or more, 100 Torr or more, or 760 Torr or more.

**[0029]** The ion or particle generator can be any device able to generate and transmit charged ions and uncharged particles, including but not limited to mass spectrometer devices and other devices able to be used as an electrospray deposition source. In an embodiment, the desired particles can be isolated or purified using the mass spectrometer prior to generating the cryo-sample. Additionally, a portion of the isolated or purified particles can be removed and analyzed while the remaining portion is used for generating the sample.

**[0030]** In an embodiment, the present invention provides a method of depositing analyte particles on a receiving surface of a sample holder comprising the steps of: a) positioning the sample holder in a sample vacuum chamber, where the sample vacuum chamber has an interior and an internal pressure; b) forming a controllable analyte beam containing charged or uncharged analyte particles in an ion or particle generator, where the ion or particle generator has an interior and an internal pressure and comprises a vacuum gate and where the vacuum gate separates the interior of the ion or particle generator from the interior of the sample vacuum chamber; c) directing the analyte beam to contact the receiving surface of the sample holder, thereby depositing analyte particles onto the receiving surface; d) reducing the temperature of the receiving surface so that the receiving surface has a first temperature of -20°C or less when contacted by the analyte beam; and e) providing a vapor to the sample vacuum chamber, where the vapor generates an amorphous solid around, under, and/or over the analyte particles deposited on the receiving surface. Optionally, the amorphous solid is generated around the analyte particles prior to the analyte particles being deposited on the receiving surface, and/or concurrently with the analyte particles being deposited on the receiving surface. A further optional embodiment comprises heating the substrate surface to a second temperature, where the second temperature is greater than the first temperature, resulting in a portion of the first solid layer undergoing a phase change to a liquid solvent.

**[0031]** In an embodiment, the methods herein further comprise measuring the mass-to-charge ratio of the analyte particles and/or filtering the analyte particles based on their mass-to-charge ratios prior to contacting the analyte particles with the receiving surface. In an embodiment, filtering the analyte particles comprises selecting a portion of the analyte particles having a mass-to-charge ratio within a desired or pre-determined range of mass-to-

charge ratios, where the analyte particles in the analyte beam only contain the selected portion of analyte particles.

**[0032]** Preferably, the interior of the ion or particle generator is separated from the interior of the sample vacuum chamber until the vacuum gate is opened and the analyte beam is directed to contact the receiving surface of the sample holder. After the analyte particles have been deposited, the vacuum gate is closed and the interior of the ion or particle generator is separated from the interior of the sample vacuum chamber so that the internal pressure of the ion or particle generator is maintained while the sample holder is removed from the sample vacuum chamber.

**[0033]** In an embodiment, the cryo-sample preparation system further comprises a sample transfer vacuum interlock comprising two vacuum gates allowing a sample to be inserted into or removed from the sample vacuum chamber without affecting the pressure in the interior of the vacuum sample chamber. In an embodiment, to remove the sample the inner vacuum gate of the interlock is opened and the sample is moved between the open inner gate and the closed outer vacuum gate. The inner gate is then closed and the outer vacuum gate is opened, which allows the sample to be removed. After the sample is removed, the outer gate can be closed and the region between vacuum gates are evacuated before the inner gate leading to the sample vacuum chamber is reopened. Suitable vacuum interlocks include but are not limited to slide gates, ball valves, and vacuum gates as known in the art.

**[0034]** In an embodiment, the present invention provides cryo-sample preparation system comprising: a) a sample vacuum chamber; b) a sample holder positioned within the sample vacuum chamber, where the sample holder comprises a receiving surface; c) a temperature control means able to provide a temperature of -20°C or less to the receiving surface of the sample holder; d) an ion or particle generator comprising a vacuum housing having an internal pressure and a vacuum gate, where the ion or particle generator is able to produce a controllable analyte beam containing charged or uncharged analyte particles, and e) a vapor source able to provide vapor to the sample vacuum chamber, where the vacuum gate is able to isolate the sample vacuum chamber from the ion or particle generator, and the sample vacuum chamber is able to produce an internal pressure different from the internal pressure of the ion or particle generator. The ion or particle generator is able to direct the analyte beam to contact the receiving surface of the sample holder, thereby depositing analyte particles onto the receiving surface. Vapor in the sample vacuum chamber is able to generate an amorphous solid around, under, and/or over the analyte particles deposited on the receiving surface, and the vapor source is able to provide vapor to

the sample vacuum chamber prior to the analyte particles being deposited on the receiving surface, concurrently with the analyte particles being deposited on the receiving surface, after the analyte particles are deposited on the receiving surface, or combinations thereof.

**[0035]** In an embodiment, the ion or particle generator comprises one or more ion or particle guides able to transmit the analyte beam to the receiving surface of the sample holder in the vacuum chamber. In an embodiment, the one or more ion or particle guides are multipoles having four or more rods, including but not limited to quadrupoles, hexapoles, or octopoles. For example, in instances where the ion or particle generator is a mass spectrometer, one end of the ion guide is attached to a cell or component of the mass spectrometer (such as an ion trap, multipole, collision cell, C-trap, an additional ion or particle guide, or an extraction region of time of flight instrument), and the second end of the ion guide is attached or indirectly connected to the sample vacuum chamber. A vacuum gate is able to isolate at least one of the ion or particle guides from the sample vacuum chamber and allow that ion or particle guide to have an internal pressure independent from the sample vacuum chamber when isolated. As used in the embodiments described herein, the vacuum gate may be any suitable valve is known in the art, including but not limited to vacuum gate valves. The ion or particle guide may be separated by the vacuum valve into multiple regions or separated to form separate guides. Similarly, different ion or particle guides may be joined together to form a single function unit having a continuous analyte path to the sample vacuum chamber.

**[0036]** In an embodiment, the analyte particles are ions and the analyte source is able to generate a controllable ion beam containing charged analyte ions, such as by electrospray ion deposition, and direct the ion beam to contact the receiving surface. In a further embodiment, the system further comprises a modified mass spectrometer that can provide purified ions to the analyte source.

**[0037]** The first temperature control means can be any cooling device as known in the art able to generate cryogenic or near cryogenic temperatures. In an embodiment, the temperature control means comprises a container in fluid communication with the sample vacuum chamber, where the container is able to provide a cooling fluid to the sample vacuum chamber and reduce the temperature receiving surface of the sample holder. The cooling fluid is preferably a cryogen, including but not limited to liquid nitrogen. In an embodiment, the cooling fluid is circulated through the sample vacuum chamber. In embodiments where the ion or particle generator further comprises a separate temperature device, the separate temperature device can similarly be any cooling device as known in the art able to cool particles leaving the ion or particle generator.

**[0038]** As used in embodiments described herein, the first temperature control means is able to provide a first temperature of -20°C or less to the receiving surface of the sample holder, preferably -40°C or less, -60°C or less, -90°C or less, -100°C or less, -130°C or less, -150°C or less, -175°C or less, -185°C or less, or -195°C or less. Preferably, the first temperature control means is able to provide cryogenic or near cryogenic temperatures to the receiving surface.

**[0039]** The second temperature control means can be the same as the first temperature control means, including but not limited to a cryostat that is used to both lower and, when switched off, allow the temperature to increase thereby raising the temperature of the receiving surface sample holder. In an embodiment, the second temperature control means is any heating device as known in the art that is able to locally increase temperature, including but not limited to a laser and an electric heater in contact with the sample holder that can be controlled with a thermostat to adjust the rate of heating and temperature of the receiving surface. In a further embodiment, the second temperature control means comprises a laser, preferably a pulsed laser, able to heat at least a portion of the receiving surface of the sample holder. Optionally, the pulsed laser has a wavelength of 360-700 nm, 700-750 nm, or 750-1,400 nm wavelength, a power or 0.10 – 1.0 mW/$\mu m^2$ or 0.50 – 1.0 mW/$\mu m^2$. Optionally, the pulsed laser is able to be rastered across the surface area of the receiving surface so as to control and limit overheating.

**[0040]** As used in embodiments described herein, the second temperature control means is able to provide a second temperature of -150°C or more to the receiving surface of the sample holder, preferably -140°C or more, -130°C or more, -120°C or more, -110°C or more, -100°C or more, -90°C or more, -80°C or more, -50°C or more, -20°C or more, -10°C or more, 0°C or more, or up to 5°C.

**[0041]** In an embodiment, the temperature of the receiving surface is increased to an elevated temperature between -130°C and -100°C, between -100°C and -90°C, between -90°C and -75°C, between -75°C and -50°C, between -50°C and -30°C, between -30°C and 0°C, or between -10°C and 0°C. In an embodiment, the temperature of the receiving surface remains at a temperature of -20°C or less, -40°C or less, -60°C or less, -75°C or less, -90°C or less, -100°C or less, -130°C or less, -150°C or less, -175°C or less or -185°C or less. In an embodiment, the temperature of the receiving surface is increased by at least 15°C, by at least 20°C, by at least 30°C, by at least 40°C, by at least 50°C, by at least 60°C, or by at least 80°C. Optionally, the temperature of the receiving surface is elevated using a heating system on the device, with photons from a laser source, combinations thereof. In a further embodiment, the temperature of the receiving surface is decreased after the heating step so

as to prepare the sample holder to be removed from the vacuum chamber and/or for cryo-EM analysis or to re-form the amorphous solid.

**[0042]** After the amorphous solid is converted to a liquid when the second temperature is applied, the amorphous solid may be re-formed by cooling the liquid to a third temperature. The cooling temperature may be applied by the first temperature control means, or in some instances by the second temperature control means. The cooling (or third) temperature is greater than the temperature provided by the second temperature control means, but may be the same or different than the first temperature provided by the first temperature control means. In an embodiment, a cooling temperature is provided, preferably by the first temperature control means, to the liquid at the receiving surface of the sample holder, where the cooling temperature is -20°C or less, preferably -40°C or less, -60°C or less, -90°C or less, -100°C or less, -130°C or less, -150°C or less, -175°C or less, -185°C or less, or -195°C or less.

**[0043]** In an embodiment, the heating of the receiving surface (such as by the second temperature control means) is performed in a heating chamber separate from the sample vacuum chamber. After deposition, the sample holder is transported from the sample vacuum chamber and into the heating chamber where the receiving surface is heated to the second temperature. The receiving surface (and sample thereon) may cooled to the third temperature in the heating chamber or the sample holder may be transported back to the sample vacuum chamber where the receiving surface and sample are cooled to form or re-form the amorphous solid.

**[0044]** In an alternative embodiment, the temperature of the receiving surface is not adjusted after the analyte particles have been deposited on the receiving surface and prior to removing the sample holder from the preparation system. In an embodiment, the temperature of the receiving surface is maintained at a temperature of -185°C or less, -175°C or less, -150°C or less, -100°C or less, -90°C or less, or -75°C or less, after particles have been deposited onto the receiving surface.

**[0045]** In an embodiment, the analyte particles are cooled prior to being inserted into the sample vacuum chamber or contacted with the vapor. The analyte particles may cooled by the temperature control means or by a temperature device that is part of or connected to the ion or particle generator. The analyte particles may be cooled to the same temperature or a different temperature as the receiving surface of the sample holder. In an embodiment, the

analyte particles are cooled to a temperature of 0°C or less, -20°C or less, -40°C or less, -60°C or less, -90°C or less, -100°C or less, -130°C or less, -150°C or less, -175°C or less or -185°C or less, prior to being inserted into the sample vacuum chamber or being contacted with the vapor.

**[0046]** In an embodiment, the temperature of the receiving surface may be adjusted after the analyte particles have been deposited on the receiving surface; after a portion of the amorphous solid has been generated on the receiving surface or around and/or over the analyte particles; or both. Preferably, the temperature of the receiving surface is raised for a brief period of time before either lowering the temperature again or removing the sample holder from the preparation system and placing into liquid nitrogen or the subsequent analytical device. In an embodiment, the temperature of the receiving surface is raised by a sufficient amount so that a portion of the amorphous solid surrounding or contacting a portion of the deposited analyte particles briefly forms a liquid. Preferably, briefly surrounding or contacting a portion of the deposited particles with a liquid may repair or mitigate damage or dehydration imparted on the particles during the deposition process, thereby improving the structural quality of the particles.

**[0047]** Optionally, the receiving surfaces and substrates described herein are electron microscopy (EM or TEM) grids as known in the art. The EM grid may comprise a metal, including but not limited to copper, rhodium, nickel, molybdenum, titanium, stainless steel, aluminum, gold, or combinations thereof as known in the art. Additionally, the EM grid may comprise a continuous film or membrane which is positioned across the top or bottom surface of the grid, or within the holes of the grid, so as to provide a solid support for the formation of the amorphous solid. Preferably, the EM grid is covered by a thin film or membrane which includes, but is not limited to, films and membranes comprising graphene, graphene oxide, silicon oxide, silicon nitride, carbon, and combinations thereof. With a grid that does not contain a film or membrane, the molecular beam intended to form the amorphous solid may pass through at least a portion of the holes in the grid without producing a suitable layer. The film or membrane should be thin enough so as to not scatter electrons. Preferably, the film or membrane has an approximate thickness or 15 nm or less, 10 nm or less, 5 nm or less, 2 nm or less, or 1 nm or less. In an embodiment, the receiving surface is an EM grid comprising a graphene or graphene oxide monolayer film or membrane positioned across the surface of the grid. In embodiments where a laser is used as the second temperature control means to heat the sample holder, the wavelength of the laser may be matched to the EM grid material (e.g., for gold EM grids, a wavelength of 532 nm may be used).

**[0048]** In an embodiment, the receiving surface comprises one or more layers of amorphous ice, and the resultant grids are able to be directly analyzed by cryo-EM. In a further embodiment, a modified Orbitrap mass spectrometer is provided that contains a lander attachment capable of directing an ion beam to a TEM grid cooled to cryogenic or near cryogenic temperatures, including but not limited to the temperature of liquid nitrogen (approximately -190°C). Beyond offering precise temperature regulation the instrument also permits deposition of molecular water, allowing formation of amorphous ice thin films as monitored by a quartz crystal microbalance in real-time. The water can be added before, during, or after the analyte particles from the mass spectrometer are deposited. The system may include a temperature control mechanism that can heat the sample (holder, grid) to temperatures greater than liquid nitrogen temperatures (e.g., -120°C) and maintain -120°C for at least 1 minute. The temperature control mechanism for cooling the sample may be used to warm the sample. Alternatively, a separate, second temperature control means may be used to warm the sample. The system integrates a retractable ion guide so that following the cryogenic landings, the mass spectrometer vacuum system can be isolated from the landing region for sample removal. A system for direct cryo-EM imaging of cryogenically landed cations of biomolecular complexes is further described below.

**[0049]** Certain aspects of the invention further include the use of mass spectrometry to purify or filter analyte particles, including but not limited to proteins, protein complexes, and cells, in the gas-phase for subsequent vitrification. Samples prepared in this way can be extracted from the mass spectrometer using a cryo-transfer sample holder and placed directly into an instrument for imaging or analysis. One implementation of this method utilizes a modified mass spectrometer that allows for gas-phase purification of analyte ions. The ions are passed over a cooled sample probe where they are deposited onto an EM sample holder and vitrified.

**[0050]** Analyte particles useful with the present invention include, but are not limited to, protein molecules, peptides, glycans, metabolites, drugs, and complexes thereof, multi-protein complexes, protein/nucleic acid complexes, nucleic acid molecules, virus particles, micro-organisms, sub-cellular components (e.g., mitochondria, nucleus, Golgi, etc.), and whole cells. In some embodiments, the analyte particles are molecular entities, single molecules, or multiple molecules complexed together through non-covalent interactions (such as hydrogen bonds or ionic bonds). Preferably, the analyte particles comprise intact biomolecules, such as intact proteins and protein complexes. In embodiments, the analyte particles have a molecular mass exceeding 500, 1,000 Daltons, 10,000 Daltons, 50,000 Daltons, 100,000 Daltons, or 150,000 Daltons.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0051]** FIG. 1 shows a cryogenic landing system with Ultra-High Mass Range (UHMR) mass spectrometer modifications in an embodiment of the invention. Panel A presents an exploded view of key components developed for cryogenic soft landings. Panel B presents a side view of the cryo-lander system with the side cover from the vacuum chamber removed for clarity. Panel C presents a top view of the landing assembly with the top vacuum cover removed for clarity. Individual components, depending on their position, may be detectable in multiple views.

**[0052]** FIG. 2 shows cryo-electron microscope images and reconstructions of room temperature versus cryogenically landed β-galactosidase cations. Panels A and B present representative micrographs of either room temperature or cryo-landed β-galactosidase cations. Panels C and D present the 2D class averages that resulted from either the room temperature or cryo-landing specimens, respectively. Panel E shows the 3D reconstruction that resulted from the room temperature landing, while panel F presents the structure obtained from the cryo-landing experiment. Panel G shows the fit of the cryo-landed model with the known high resolution ribbon structure.

**[0053]** FIG. 3 shows a comparison of angular particle orientations resulting from either room temperature (A) or cryogenically (B) landed β-galactosidase cations. Note the room temperature landing sample resulted in a highly preferred orientation while the cryo-landed sample does not.

**[0054]** FIG. 4 shows representative micrographs of the resulting room temperature and cryo-landed proteasome particles. As in the β-galactosidase experiment, the quality of the particles cryogenically landed on ice is substantially better than those landed at room temperature, with more detail apparent in both the 2D class averages (panels C and D) and the 3D reconstruction (panels E and F).

**[0055]** FIG. 5 shows hydration level of proteins in solution. Protein structure is a function of the amino acid sequence as well as the absorbed water molecules.

**[0056]** FIG. 6 shows the number of absorbed waters on protein molecules. Circles = absorbed waters in solution, includes internal and hydration shell waters. Squares = absorbed waters in gas phase drift tube experiments (4-5 fewer waters are observed in the gas phase drift tube experiments). Triangle = maximum number of absorbed waters observed in the native mass spectra. However, while not shown, many ions had fewer absorbed waters.

**[0057]** FIG. 7 shows cryo-EM 3D reconstructions of β-galactosidase imaged under similar conditions. Panel A shows a 3D reconstruction using plunge freezing of the sample. Panel A shows a 3D reconstruction using soft landing without the use of the additional methods of the present invention. The resulting lower resolution is likely because of heterogeneity of the individual molecules because of varying amounts of hydration or other damage during landing.

**[0058]** FIG. 8 shows β-galactosidase soft landed under similar conditions. Panel A shows a more conventional setup in which the TEM grid is maintained at cryogenic temperatures throughout landing and imaging. Panel B shows a sample prepared by warming the TEM grid after initial landing/coating in amorphous ice, allowing the molecules to rehydrate, followed by cooling again to cryogenic temperature, plunge freezing, and imaging.

**[0059]** FIG 9 shows a phase diagram for water (see Feistel et al., J. Phys. Chem. Ref. Data 1 2006; 35(2):1021–1047).

**[0060]** FIG 10 shows time necessary for the conversion of vitreous to cubic ice (see Dowell et al., Nature 1960, 188: 1144–1148).

**[0061]** FIG 11 shows a breadboard instrument in an embodiment of the present invention, where the instrument includes a TEM grid, grid holder, and pulsed laser. Proposed configuration allows for TEM grid (sample) to be exposed to pulsed laser while at cryogenic temperatures to induce localized heating, warming, rehydration of particle (sample), followed by refreezing under cryogenic conditions for further processing and imaging via cryo-EM. (Note: that the PEEK alignment housing is pulled back from the grid so the laser is visible).

**[0062]** FIG. 12 shows a breadboard instrument showing a sample vacuum chamber in an embodiment of the present invention. Panel A shows the TEM grid probe moved into position for ions to be deposited from the mass spectrometer (far right, not shown). Panel B shows the TEM grid probe retracted from the ion or particle generator (modified mass spectrometer) and the water doser moved into a position to precisely deposit amorphous ice onto the grid. Water can be added before landing, during landing, or after landing. Panel C shows the laser moved into position to melt, hydrate, and refreeze the sample. This also shows the vacuum gate to the sample vacuum chamber (far right) that can isolate the sample vacuum chamber from the ion or particle generator (not shown).

DETAILED DESCRIPTION OF THE INVENTION

**[0063]** Overview

**[0064]** Mass spectrometry technologies are able to analyze protein abundance, modification states, interaction partners, or even biochemical pathways in which proteins function. Multiple mass spectrometry technologies including ion mobility, chemical crosslinking, covalent labeling, and dissociation technologies, such as surface-induced dissociation, have been developed with the intent of gaining further insight into the structure of biomolecules. Many of these technologies are increasingly used in combination with cryo-EM to obtain complete structural characterization. Fully integrating mass spectrometry with cryo-EM would greatly accelerate the pace at which new structures could be determined. To that end, the examples below describe the use of soft-landing mass spectrometry in order to produce samples for cryo-EM and provide a unique instrument enabling the production of such samples.

**[0065]** A landing apparatus (see, for example, **Figure 1**) and method are described that allow for temperature control of a TEM grid within a mass spectrometer environment, formation of amorphous solid, and a means to remove the sample from vacuum without contamination. Such systems demonstrate considerably improved structural resolution, as evidenced by cryo-EM imaging of cryogenically landed protein–protein complex cations. In addition to improving structural quality, this system also provides a much more diverse particle orientation than seen with room temperature landing and allows for the direct coupling of mass spectrometry with cryo-EM.

**[0066]** Example 1 – Methodology

**[0067]** **Sample Purification.** The purification of both β-galactosidase and the 20S proteasome core particle has recently been described (Salome et al., Analytical Chemistry 2022, 94(50): 17616-17624). The procedures are additionally summarized below.

**[0068]** **β-Galactosidase Preparation**. β-Galactosidase (from *Escherichia coli*, G3153-5MG) was purchased from Sigma Aldrich and prepared at 1 mg/mL in 100mM ammonium acetate. 10μL of the buffered protein was added to an additional 300μL of buffer and placed in an Amicon centrifugal filter. The sample was centrifuged (Thermo Scientific Sorvall Legend Micro 21R) at 14,000 g for 10 minutes at 4°C. Buffer which passed through the filter was discarded and an additional 400 μL of buffer was added to the sample for another round of washing using the same centrifugal settings. This wash process was repeated three times total. To obtain the final sample, the filter was inverted and centrifuged at 2000 g for 1 minute and diluted with 100mM ammonium acetate to a final concentration of roughly 1 μM.

**[0069]** **20S proteasome core particles**. Thermoplasma acidophilium expressed in *E. coli* BL21(DE3) was provided by University of California, San Francisco, in uranyl acetate (1% solution). 100µL of the stock solution was transferred to an Amicon spin filter and centrifuged at 10,000 g for 10 min at 4 °C. Three rounds of purification were done with 400 µL each of 100 mM ammonium acetate followed by centrifugation at 10,000 g for 10 min at 4 °C with flow through being discarded. Final sample was obtained by inverting the Amicon filter and centrifuging at 2000 g for 1 min at 4 °C.

**[0070]** **Mass Spectrometry and Soft Landing**. Mass spectrometry experiments were performed on a modified Thermo Scientific Q-Exactive UHMR Hybrid Quadrupole-Orbitrap mass spectrometer. Borosilicate glass capillaries were pulled in-house using a model P-2000 laser-based micropipette puller (Sutter Instrument, CA) to an emitter inner diameter of 1 to 5 microns. A platinum wire placed within the capillary provided continuity between the mass spectrometer ESI power supply and the solution being sprayed.

**[0071]** All full scan MS1 experiments were conducted with an ESI voltage of 1.1kV to 1.5kV, mass resolving power of 6250 at m/z 400, inlet capillary temperature of 200°C, and no in-source trapping. For landing experiments, the Orbitrap was not employed, and the ions were not stopped within the c-trap. To prevent trapping, all gas flow into the c-trap was ceased. The following DC gradient was placed on the ion optics from the inlet of the mass spectrometer to the TEM grid: Source DC offset 0V, injection flatapole 1V, inter flatapole lens 1V, bent flatapole 1V, transfer multipole 1V, C-trap entrance lens 0V, HCD field gradient 0V, HCD cell DC offset -4.5V, HCD cell exit lens 0V, and the TEM grid -1V. A wide mass filter isolation of 8,000–18,000 m/z was employed. Proteins were landed on ultra-thin carbon over lacy carbon grids (CLC400Au25-UT, EM Resolutions, UK) which were first glow discharge for 30 seconds.

**[0072]** **Cryo-EM Imaging and Data Processing**. Movies for both the room temperature and cryogenic landing were collected at 200 kV on a Thermo Fisher Scientific Glacios TEM controlled by SerialEM (Mastronarde, D. N., J Struct Biol 2005, 152(1): 36-51). Movies were collected for the room temperature sample and for the cryo sample using a Falcon 3 camera operated in counting mode (calibrated pixel size - approximately 200 Feet). Data sets were collected employing a total dose of approximately 97 e/Å$^2$ over about 67 frames per movie. Images were collected with a defocus range of approximately 0.5 to 2.0 µm.

**[0073]** Collected movies were imported into *cis*TEM 2.0 for processing (Grant et al., eLife 2018, 7: e35383). The standard workflow included motion correction followed by constant transfer function (CTF) parameter estimation. Only micrographs with a detected

CTF fit resolution better than 1.0 inch were kept for further processing. Next, automated sample picking, within *cis*TEM, was employed followed by 2D classification. Particles initially picked for the room temperature sample and the cryo sample were used to construct good class averages displaying sufficient particle features. *Ab initio* reconstructions were created and further refined via auto-refinement imposing D2 symmetry.

**[0074]** Example 2 - Results

**[0075]** To test the hypothesis that cations of protein–protein complexes could be preserved and prepared for cryo-EM using MS, the landing apparatus shown in **Figure 1** was constructed.

**[0076]** Components are labeled with numbers 1 through 26 and a prefix of a, b, or c depending on the corresponding view. The components of the apparatus in this example are as follows: 1) Autogrid assembly (i.e., a TEM grid), 2) Autogrid top plate, 3) Autogrid transfer assembly body (sample holder), 4) copper set screw, 5) copper heatsink, 6) PEEK liquid nitrogen feed, 7) PEEK liquid nitrogen and nitrogen vapor return, 8) PEEK cold block insulator, 9) cryo-temperature sensor, 10) higher-energy C-trap dissociation (HCD) cell exit lens, 11) cryo-lander vacuum chamber, 12) modified vacuum housing of UHMR, 13) vacuum gate to the sample vacuum chamber, 14) exposed HCD cell, 15) nitrogen gas feed to UHMR C-trap, 16) linear actuator for insertion of TEM grid holder into mass spectrometer, 17) UHMR C-trap, 18) spring loaded clamp, 19) linear actuator for TEM grid holder release, 20) sample transfer vacuum gate, 21) linear actuator for TEM grid cover, 22) turbo molecular pump, 23) vent gas inlet valve, 24) pressure relief valve, 25) Dewar containing or full of liquid nitrogen, 26) micro balance, 27) PEEK water vapor inlet line, 28) vacuum gauge, and 29) temperature sensor. These components are illustrated in one or more views **Figure 1**, **panels A-C**.

**[0077]** The foremost design consideration for this device was to ensure sufficient thermal contact to the TEM grid so that even under the vacuum environment heat could be removed fast enough to allow formation and maintenance of amorphous ice—at least $10^6$ degrees Kelvin per second. Since the ultimate destination of the TEM grids was a Thermo Fisher cryo-EM system outfitted with an autoloader, each grid was initially mounted in an autogrid ring and c-clip (Thermo Fisher Scientific part numbers 1036173 and 1036171, respectively). To interface with the TEM grid, a sample holder was designed which not only enabled excellent thermal contact to the grid but also provided sufficient thermal mass to prevent rapid temperature swings at the grid. **Figure 1, items a1 through a4** presents an exploded view of the grid transfer assembly. Note the total mass of a single TEM grid is ~ 1 mg, while the grid transfer assembly weighs in at 8 grams—a ~$10^4$ fold difference. Aside from the

mass difference, the grid transfer assembly affords quick energy transfer to the grid by sandwiching the Autogrid assembly (**Figure 1, a1**), with a threaded copper set screw (**Figure 1, a4**), against the holder top plate (**Figure 1, a2**) that slides through a slot in the grid transfer assembly body (**Figure 1, a3**).

**[0078]** Having developed a grid holding device, a cooling apparatus was constructed that would precisely regulate the temperature of the grid transfer assembly to as low as -190 C at pressures ranging from atmosphere to vacuum. The central framework of the temperature regulator is a copper heat sink that has within it an open channel for the passage of nitrogen (**Figure 1, a5**). To isolate the heat sink thermally and electrically, PEEK bushings were fabricated to go between the cold block and linear actuating rod (**Figure 1, a8**) as well as at the attachment points of the liquid nitrogen feed and return lines (**Figure 1, a6 and a7**). Also attached to the temperature regulator's copper heat sink are resistive heating element (HT15W, ThorLabs, Newton, NJ) (**Figure 1, a29)** and a LakeShore DT-470 series silicon diode (Lake Shore Cryotronics, Westerville, OH) (**Figure 1, a9**), to adjust and track temperature, respectively. Finally, the temperature regulator module is electrically connected to an RBD 9103 autoranging picoammeter (RBD instruments, Bend, OR) so that ions landing on the TEM grid surface can be detected.

**[0079]** An additional objective is to embed particles within amorphous ice and other amorphous solids. One way to do this is to use background gas present in the vacuum system, i.e., contaminating water vapor. To obtain a more controlled deposition, a third module was added which offers the ability to calibrate the rate of ice formation. This component is inserted between the copper heat sink and the grid transfer assembly and is based on a cryogenically cooled quartz crystal microbalance constructed in house (**Figure 1, a26**). Fashioned with a copper housing, the microbalance forms an extension of the cold block so that the grid transfer assembly continues to be sufficiently cooled. Briefly, as vapor or ice condenses onto the cryogenic temperature quartz surface, the frequency at which it oscillates changes. The change in frequency directly corresponds to the thickness of ice that has built up on the surface of the microbalance. These frequency changes are sufficiently sensitive to permit preparation of ice thickness to within a single nanometer. Note that the quartz crystal microbalance device is used to calibrate the rate of water vapor introduction prior to ion beam deposition and while the grid transfer assembly is removed. Once this calibration is complete, the grid transfer assembly may be put in place for landing experiments. Water vapor, for ice formation, is introduced from the head space above water (American Society for Testing and Materials Type 1) contained within a glass vial which was previously evacuated by the mass spectrometer pumping system. The vapor is introduced

into the HCD cell vacuum chamber through PEEK tubing (**Figure 1, c27**) and regulated with a pin valve.

**[0080]** The cryo-lander is housed in its own vacuum chamber (**Figure 1, b11**) and aligns the TEM grid with the last ion optic of the mass spectrometer (exit lens of the HCD cell) (**Figure 1, a10**). A Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific Orbitrap UHMR) was modified to accept this device by shortening the vacuum chamber which houses the HCD cell (**Figure 1, c12**) to minimize the distance between the exit lens of the HCD cell and the vacuum gate that separates the cryo-lander from the mass spectrometer. To reduce background contaminants that could form on the cryogenically cooled grid surface, the overall operating pressure of the central and rear regions of the mass spectrometer was lowered. To this end, the original, encased HCD cell was modified by removing the outer enclosure and ceased the flow of nitrogen into the cell (**Figure 1, a14**). Doing so reduced pressure from $10^{-4}$ bar to $10^{-6}$ bar, in effect removing the function of mass analysis in the Orbitrap, as C-Trap and HCD cell pressures are too low to stop ions. To restore the ability to put ions in the Orbitrap, a new gas line was run into the C-Trap (**Figure 1, a15**), pressurizing the C-Trap (**Figure 1, a17 and b17**) with nitrogen only when mass analysis is required, but stopping the gas flow for all landing experiments. Finally, the HCD cell RF and DC, axial DC gradient, and accompanying exit lens were operated using Modular Intelligent Power Sources (GAA Custom Electronics, Kennewick, WA).

**[0081]** This setup enables the process of cryo-landing. To load the receiving grid, the cryo-lander was vented and the device opened by first retracting the horizontal actuator (**Figure 1, c16**) until the plunger of the spring-loaded clamp (**Figure 1, a18 and c18**) intersects with the fully inserted center most vertical linear actuator (**Figure 1, b19**). The sample transfer vacuum gate (**Figure 1, b20**) was opened to insert the grid transfer assembly through the bottom of the vacuum chamber up to a spot between the cold block and spring-loaded clamp, the horizontal actuator moved forward 1cm to close the gripper, and the grid transfer assembly held firmly in place. With the grid transfer assembly in place, the center most vertical actuator was retracted and the outer most vertical linear actuator inserted (**Figure 1, b21**), which places a PEEK cover over the grid to prevent contamination during the cooling process.

**[0082]** To return to vacuum a turbo molecular pump was utilized (Turbovac 350i, Leybold, Cologne, DE) (**Figure 1, b22**) to achieve a vacuum of $1x10^{-5}$ Torr. Once at vacuum the flow of liquid nitrogen through the cold block begins. Control of the nitrogen flow and over all temperature regulation is provided though a cryogenic temperature controller (CTC100, Stanford Research Systems, Sunnyvale, CA). After the cryo-lander module reaches the

desired temperature (such as -190°C), the outer most vertical linear actuator is raised, releasing the grid transfer assembly cover, opening the vacuum gate between the lander and mass spectrometer (**Figure 1, c13**) and the module is inserted using the horizontal actuator (**Figure 1, C16**) into the mass spectrometer such that the TEM grid is within one to two millimeters of the exit lens of the HCD cell. Note, the mass spectrometer ion beam has previously been tuned and directed in the Orbitrap and cryo-lander systems. Then, the ion current impinging on the grid is checked using the picoammeter to ensure the ion current is around 0.1 nanoamps. Landing typically takes 10 to 20 minutes, with the grid in place behind the HCD cell.

**[0083]** Once landing is complete, the extraction process is as follows: the horizontal linear actuator is retracted and the outer most vertical actuator is lowered to return the cover to the front of the grid. Next, the vacuum gate between the lander and mass spectrometer is closed, separating the lander from the mass spectrometer, and the cryo-lander pumping system is turned off. At the same time, the nitrogen gas inlet valve (**Figure 1, b23 and c23**) is opened to flood the cryo-lander chamber with nitrogen vapor from the headspace of a liquid nitrogen Dewar. Once the pressure inside this chamber reaches 1 psi, a safety valve (**Figure 1, b24**) opens to prevent further buildup of pressure. From this point on, the nitrogen vapor is flowing, and the liquid nitrogen continues to flow through the cold block to provide cooling. Continuous nitrogen vapor flow keeps the chamber at a positive pressure, preventing contamination from atmospheric water vapor. A small Dewar (**Figure 1, b25**) of liquid nitrogen is placed below the sample transfer vacuum gate, which provides a continuous vapor of nitrogen pushing out atmosphere in the region and a pool of liquid nitrogen in which the grid transfer assembly will be deposited. The center most vertical linear actuator is then lowered, the sample transfer vacuum gate opened, and then the horizontal actuator retracted until the plunger of spring-loaded clamp intersects with the center vertical actuator opening the clamp and releasing the grid transfer assembly which drops into the liquid nitrogen, with the sample preserved at cryogenic temperature.

**[0084]** To test the cryo-lander, β-galactosidase was selected as its structure is well understood and it serves as a common cryo-EM reference complex. Native electrospray of a β-galactosidase solution was performed in ammonium acetate buffer. Using the setup described above, except set at room temperature, cations (from the entire charge envelope, z ~ 42-48) of β-galactosidase were deposited onto amorphous carbon-coated TEM grids for 15 minutes with an average energy of 3–4 eV per charge. The grid was removed as described above and kept submersed in liquid nitrogen until it was placed into a 200 keV Thermo Fisher Scientific Glacios Cryo-EM system.

**[0085]** **Figure 2** depicts a comparison between the room temperature landing and the cryogenic landing using the device described above. Panels A and B present representative micrographs of the room temperature and cryo-landed particles, respectively. It is immediately evident that the particles shown in Panel A are free of ice, and thus the contrast of the particles is high. In Panel B, by contrast, there appears to be a thin film of what appears to be ice in addition to the particles, which therefore exhibit lower contrast. The formation of this thin film was likely due to contaminant water vapor in the vacuum system, which condensed while the particles were deposited. The ice formation module of the instrument had not yet utilized when these data were collected. Results using that method of ice making are detailed below.

**[0086]** Panels C and D of **Figure 2** show 2D class averages of particles from the respective landing conditions. The class averages from the room temperature landing lack features, appear to be damaged, and to have a strong preferential orientation. The 2D class averages obtained from the cryo-landing demonstrate more features and a much wider range of orientations (**Figure 3**). Similarly, the 3D reconstruction in Panel F shows better conserved features than the reconstruction derived from room temperature landing in Panel E. Indeed, the Panel E reconstruction is so featureless as to at best represent only the molecular envelope of β-galactosidase. In the 3D reconstruction derived from cryo-landing, however, the contour generally follows the known structure of β-galactosidase (Panel G).

**[0087]** The ability to obtain a more controlled water deposition and ice formation was tested next, along with a test landing of another sample. The cryogenically cooled TEM grid was first coated with ~ 10 nm of amorphous ice by opening the water vapor valve (see above) and waiting ~ 5 minutes (i.e., the device was calibrated using the QCM for a deposition rate of ~ 2 nm ice per minute). The water vapor valve was then closed and immediately followed by deposition of the entire charge envelope of proteosome cations for 15 minutes. At this point the TEM grid was removed from the instrument as described above and placed into the EM and kept cold the entire time. A room temperature control deposition of the same proteasome cations was also collected.

**[0088]** Panels A and B of **Figure 4** display representative micrographs of the resulting room temperature and cryo-landed proteasome particles. As in the previous β-galactosidase experiment, the quality of the particles cryogenically landed on ice is substantially better than those landed at room temperature, with more detail apparent in both the 2D class averages (panels C and D) and the 3D reconstruction (panels E and F). Also mirroring the previous experiment, despite demonstrating some protection with the cold, amorphous ice-coated surface, the quality of the cryogenically landed particles is still lower than would be expected

in a conventionally prepared proteasome specimen suggesting the protection could be improved. Interestingly, several of the 2D class averages of the cryo-landed proteasome cations show what appear to be droplets or water build-up around the edges. Note mass spectral measurements were acquired (data not shown) and do not indicate the presence of sufficient water molecules to explain the droplets. It was concluded that the water must build-up around the particles post-deposition. This phenomena was much less pronounced but still present in the β-galactosidase experiment as demonstrated by the middle left class average of **Figure 2, panel D**.

**[0089]** Unlike the β-galactosidase experiment, when comparing the angular orientation distributions of the proteasome particles in both the room temperature and cryogenically landed experiments there is no obvious difference. Both samples exhibit the classic preferential orientation towards top and side views seen in traditional proteosome reconstructions. It is unclear whether this is a sample specific effect, or related to the different nature of ice formation in the two experiments.

**[0090]** Example 3 – Sample Hydration

**[0091]** Protein structure is a function of not only the amino acid sequence but also the absorbed water molecules. Proteins typically imaged by cryo-EM include 0.32 g of water / g protein. Protein hydration data from the literature is summarized in **Figure 5** (Lee et al., J. Am. Chem. Soc.1998, 120:11758; Rodriguez-Cruz et al., J. Am. Soc. Mass. Spectrom. 1997, 8:565; Kuntz et al., Adv Protein Chem 1974, 28:239; Steinberg et al., Phys Chem Chem Phys 2007, 9:4690; Yang et al., Biochem 1979, 18:2654; Doster et al., Biophys J 1986, 50: 213; Steinbach et al., PNAS 1993, 90: 9135; Bull, H.B., J. Am. Chem. Soc.1944, 66: 1499; Wyttenbach et al., Int J Mass Spec 2005, 240:221; and Fye et al., J. Am. Chem. Soc.1998, 120:1327).

**[0092]** As shown in **Figure 6**, large proteins will have thousands of absorbed water molecules. In the present work, mass spectrometry showed that under native mass spectrometry conditions for β-galactosidase, ~8,000 water molecules were observed based on the mass difference from the unhydrated protein at 466 kDa. However, about half of the protein ions contained fewer than a full complement of waters seen in solution (not shown).

**[0093]** Although the literature states that extensively hydrated gas-phase biomolecule ions can be produced with a sufficient number of water molecules attached that they exhibit solution-phase structures (see Rodriguez-Cruz et al., J Am Soc Mass Spectrom. 1999, 10(10): 958–968), altered structures have been observed after soft landing. For example, Brodmerkel et al. (Protein 2023, J42: 205–218) states that "*Vacuum exposure affects protein*

*structures… which can ultimately lead to a compaction, manifested as a decrease of their volume, CCS, and surface area… Upon rehydration, back in solution, interactions between the residues and the solvent allow the protein structures to relax and expand, potentially reverting those changes.*" Moreover, Esser et al. (PNAS Nexus 2022, 1(4): pgac153) states that "*maintaining hydration, achieving controlled rehydration, or landing in a liquid environment, the latter recently demonstrated by Westphall et al. (57), has the potential to preserve or restore protein structures closer to those in the native environment.*"

**[0094]** An issue is how to rehydrate protein and other ions that have been landed on TEM grids. In general, two basic ways to examine hydrated proteins in the gas phase include either leaving water on the protein ion when it is produced or adding water to a dehydrated protein (Jarrold, Martin, Accounts of Chemical Research 1999, 32(4): 360-367). Ion mobility drift cells have been used to hydrate gas phase protein ions. As shown in **Figure 2**, even under the best conditions drift cells are only able to reach 20-25% of the number of waters found in solution. As noted above, incomplete, partial, and variable hydration influences the 3D structure of the proteins. The shortcomings of the drift cell indicate that sufficient hydration of gas phase ions cannot be achieved during landing. As a result, a method and related system for hydrating particles (proteins) after landing is needed.

**[0095]** Example 4 – Hydration of plunge frozen samples v. soft landed samples

**[0096]** The number of absorbed waters influences the molecular structures and the homogeneity of particles. Therefore, absorbed waters affects (limits) imaging resolution. For example, **Figure 7** shows β-galactosidase imaged with the same microscope, using the same 3D reconstruction techniques. Although the overall shape and size of the molecule is the same, **Figure 7**, **panel A** was plunge frozen in the traditional manner and is much higher resolution. The soft-landed reconstruction, shown in **Figure 7**, **panel B**, suffers from lower resolution resulting in a blurry image. The reduction in resolution from **Figure 7**, **panel A** to **Figure 7**, **panel B** can likely be attributed to structural heterogeneity, due at least in part to variation in hydration.

**[0097]** Another soft-landing limitation is potential changes to the particle structure caused by the impact of the particle on the surface. While it is believed that the 2-8 eV/charge kinetic energy (90-350 eV for the of β-galactosidase shown in **Figure 7, panel B** used during soft landing is insufficient to break bonds, structural changes may be possible. Depending on the orientation of the ion when it impacts the surface, different structural changes could occur. Again, this leads to particle heterogeneity. One option for increasing

homogeneity is to "repair" the samples after landing (and coating in amorphous ice) through the introduction of water molecules.

**[0098]** One option for limiting damage during soft landing is to leverage a TEM grid coating. Several groups have landed ions onto TEM grids coated with a matrix such as glycerol or polypropylene glycol (Siuzdak et al., Chemistry & Biology 1996, 3: 45-48; Ouyang et al., Science 2003, 301:1351-1354; Nie et al., Anal Chem 2004, 15:1874-1884; and Salome et al., Anal Chem 2022, 94:17616-17624). It is believed these surface coatings help to hydrate the landed molecules or possibly prevent further dehydration. However, these coatings are not compatible with cryo-EM. Therefore, the imaging resolution is inherently limited. Accordingly, an alternative way to hydrate landed particles is need that is compatible with cryo-EM.

**[0099]** Example 5 – Warming using an electric heater

**[0100]** In one embodiment, the temperature of the sample grid is increased after the particles are landed. Heating after landing is believed to enable hydration of particles via absorption of water molecules from the surrounding ice layer. Hydration may be accompanied by a structural change from a higher energy state to a lower energy conformation. Warming is achieved while the sample grid is in the vacuum chamber.

**[0101]** In an embodiment, a sample (e.g., β-galactosidase) is deposited onto a sample grid (exemplary duration of 20 min, exemplary temperature of -187°C). Ice is then deposited on the sample (exemplary duration 40 seconds, exemplary partial pressure of ~$3.0 \times 10^{-5}$ Torr) resulting in a layer of ~20 nm thick of amorphous ice. The amorphous ice coated sample is then heated to a temperature at which point a portion of the amorphous ice becomes accessible for rehydration of the particle (i.e., a protein) (exemplary target temperature of -120°C, exemplary heating time of ~4 min, exemplary hold time of 1 minute). The sample is then cooled back to a cryogenic temperature at which amorphous ice is formed (exemplary temperature of -187°C). The sample is subsequently plunge frozen in liquid nitrogen and imaged with cryo-EM.

**[0102]** In previous work, amorphous ice was devitrified using an electric heater (Cyrklaff et al., Ultramicroscopy 1994, 55:141); however, the resulting ice was crystalline. Here, the electric heater warms the sample to allow the sample to hydrate. Once hydrated, the sample is then rapidly cooled to vitrify the ice creating an amorphous ice layer (i.e., non-crystalline).

**[0103]** 3D reconstructions β-galactosidase prepared using the standard soft-landing method (i.e., maintaining at -187 °C) and the warming method (i.e., raising the temperature

to -120 °C) are shown in **Figure 8.** The warmed sample in **Figure 2**, **panel B** shows a marked improvement in resolution when compared to the sample that is maintained under cryogenic conditions shown in **Figure 2**, **panel A**. Given these results, it is believed that warming and refreezing the sample increases particle homogeneity, which is important given that high-resolution 3D reconstruction requires atoms to be in the same place/orientation across >10,000 particles contained in a typical sample. One possibility is that the warming allows the reincorporation of some of the water molecules that are lost before, during, and possibly after gas phase landing on the substrate (e.g., a TEM grid).

**[0104]** Example 6 – Ice phase and preventing sublimation

**[0105]** The warming method could be achieved by including electric heaters coordinated with a cryostat, which would enable finer control over heating (rate) and the target temperature. Further, once the sample is warmed, limiting the formation of crystalline ice and sublimation of water will be important. For the latter, the ice is there to prevent dehydration of the sample particles when exposed to vacuum as well as providing a minimum amount of electron radiation protection. For the form, the phase/structure of the ice must be amorphous (vitreous) and not crystalline in order to allow electrons to penetrate and reach the sample during cryo-EM.

**[0106]** Operating conditions for soft-landing include a vacuum chamber pressure of $1x10^{-5}$ Torr (1.4 mPa). At this pressure the ice needs to be maintained at a temperature of -100°C or less, or else sublimation will occur as shown in **Figure 9**. Essentially, the sublimation temperature is a consideration (boundary condition) used to identify operating parameters for warming the sample to allow particle rehydration and reconfiguration to a lower energy state. Attempts to warm the sample to -100 °C and -50 °C were unsuccessful, resulting in sublimation of the ice and loss of the sample.

**[0107]** The warming could be done at higher pressure (e.g. ambient pressure). This would allow the sample to be warmed to room temperature without sublimation of the ice. However, crystalline ice forms if the sample is cooled too slowly. To promote amorphous ice formation, it is proposed raising the grid temperature high enough to promote hydration (e.g., 5°C) and then plunge freezing the grid. Alternatively, jets of ethane could be used to quickly cool the grid.

**[0108]** One limitation of warming while the sample is held at vacuum (e.g. <4.6 Torr) is the upper temperature to which the sample can be heated before the ice sublimates (see **Figure 9**). Once sublimated, the ice cannot hydrate the particles. Therefore, an alternative embodiment would be inducing rehydration at higher pressures. Potential options for

achieving these conditions could include an isolated sample landing chamber in which the pressure can be increased (i.e., separated from the mass spectrometer vacuum chamber or ion or particle generator), or a wholly separate instrument built specifically for heating. It can be done in the presence of various inert purge gases such as nitrogen, helium, and carbon dioxide. It could also be done in the presence of a vapor such as water vapor.

**[0109]** Heating could be accomplished by warming or with a laser. In the case of slower heating, it may be advantageous to raise the pressure (e.g., by introducing nitrogen, helium, or some other inert gas) in the sample vacuum chamber to >4.6 Torr, >100 Torr, or >760 Torr. At these pressures, the ice will not sublimate but instead turn to liquid water. After waiting a suitable length of time (e.g. 1-100 μs, 100-1000 μs, 1-1000 ms, or 1-100 s), particles will hydrate. Then, the sample is cooled in such a way to vitrify the water forming amorphous ice. This can be accomplished by rapid cooling through plunge freezing or jets of liquid ethane or liquid nitrogen. In the case of rapid heating with a laser, plunge freezing may not be necessary. The thermal mass of the grid, which is held at cryogenic temperatures, may be sufficient to rapidly cool the small area heated by the laser. Such rapid cooling can form amorphous ice directly without the need for plunge freezing.

**[0110]** A temperature sensor attached to the sample holder may not exactly report the temperature of the sample itself. Therefore, it may be difficult to determine how high the temperature of the sample holder can be raised before sublimation of the ice occurs. A quartz microbalance could be used to monitor the change in mass of the sample holder. This mass loss could then be correlated to sublimation of the ice. The temperature of the sample holder could be raised until the rate of mass loss reported by the quartz microbalance reached a threshold. This would represent the maximum sample holder temperature that could be achieved with the sublimation of the ice.

**[0111]** Another option is to exploit the idea that only warming the grid for a very short time does not allow enough time for ice to crystallize. **Figure 10** shows that ice under vacuum can remain vitreous if it only remains at higher temperatures for a short time. Extrapolating to near room temperature, which may or may not be accurate, means that ice remains vitreous if held at 5°C for $<10^{-27}$ s. This hold time would be very difficult to achieve, and as a result alternative methods were sought.

**[0112]** Example 7 – Laser Melting

**[0113]** Pulsed lasers have been used to heat TEM grids rapidly for time-resolved electron microscopy experiments (Voss et al., Struct. Dyn. 2021, 8:054302; Voss et al., Chem Phys Lett 2021, 778:138812; Bongiovanni et al., Frontiers Mol Biosci 2022,

9:1044509; Harder et al., Acta Cryst 2022, D78:883; Harder et al., Nature Comm 2023, 14:5649).

**[0114]** In these studies, a 532 nm laser was used with a 24 µm spot size to heat a small region of the TEM grid. Using 63-205 mW, 10-50 µs laser pulses, the grid was able to be heated to ~27°C after which the ice rapidly cooled and revitrified to form amorphous ice. The green laser light is absorbed by the gold TEM grid which in turn heats the ice and sample molecules. The thermal mass of the grid relative to the small region heated allows it to cool very rapidly, vitrifying the ice.

**[0115]** These studies used the laser heating to study molecular dynamics at µs time resolution. In the present invention, a pulsed laser is used to heat the grid, allowing the molecules to rehydrate, and allowing the grid to cool rapidly. Rather than target specific regions of the grid with laser pulses of different parameters (e.g., power, duration, or spot size) as described in the above studies, the present invention preferably applies the same parameters on all target areas of the grid so as to achieve consistent thawing, hydrating, and refreezing of the particles.

**[0116]** An instrument to enable the laser melting method is shown in **Figure 11** and **Figure 12**. Ultimately, the instrument allows for rastering the pulsed laser across the surface of the sample holder (TEM grid). Rastering simultaneously allows for localized warming while still enabling rapid cooling of the ice, thereby enabling rehydration of the particle and avoidance of crystallization/sublimation of the ice (i.e., retaining amorphous/vitreous ice). The sufficient thermal mass of the grid, sample, and ice facilitates this approach.

**[0117]** In a potential implementation of the pulsed laser for warming the sample, all the steps are performed in the soft-landing chamber of the mass spectrometer. In so doing, it is believed this will minimize grid and sample contamination. As shown in **Figure 12**, **panel A**, the TEM grid probe **41** is moved into position toward the vacuum gate to the sample chamber **43** to receive ions from an ion source (not shown), such as a mass spectrometer device. A water doser **46** is able to provide the vapor to form the amorphous ice. The temperature of the TEM grid is able to be controlled through the use of cryogenic cooling lines **42**. After ion deposition, the TEM grid probe **41** can be positioned for precise ice deposition by retracting sample holder **43** as shown in **Figure 12**, **panel B**. A pulsed laser **48** is then used to heat the TEM grid **44**, thereby locally heating and melting ice for a set period of time, allowing the particles to hydrate, after which the sample is (i.e., the pulsing of laser will cease) allowing for refreezing (amorphous ice formation) on the particles (shown in **Figure 12**, **panel C**). Once refrozen, the sample is able to be removed through the sample

transfer vacuum gate **50** and plunge frozen in a cryogenic liquid **47**, such as liquid nitrogen, contained in a stainless steel Dewar **45** (see bottom of **Figure 12**, **panel A**) or moved into the imaging microscope (not shown).

**[0118]** Possible operating conditions will be informed by ice phase and sublimation considerations as described in Example 6 above. Potential operating conditions may include a pulsed laser with controlled spot size so as to control the size of the area heated on the TEM grid. Exemplary laser spot size is between 5 µm and 1 mm or between 10-100 µm. Laser wavelength is determined by the grid material (e.g., gold), the grid coating (e.g., carbon), or the water deposited thereon. Exemplary wavelengths absorbed by these materials include 360-700 nm, or 700-750 nm, or 750-1,400 nm. Laser power is determined by sufficiency to warm, heat, and melt the ice in the area of interest. Exemplary laser power will likely be between 0.10 – 0.5 mW/µm$^2$. The spot (area of interest) needs to be warmed to a sufficient temperature to allow water to be absorbed and the landed molecules to relax to a low energy conformation. Exemplary increases in temperature are likely >1 ºC and < 210ºC or between 1-50 ºC.

**[0119]** An alternative approach is inducing rehydration under cryogenic conditions at higher pressures (e.g., ambient pressure). Potential options for achieving these conditions include an isolated landing chamber in which the pressure is increased (i.e., separated from the mass spectrometer vacuum chamber, or a wholly separate instrument built specifically for laser heating).

**[0120]** A consideration that may inform the laser power and pulse width is avoiding unwanted rotation particles during reheating and rehydration. For a complete/comprehensive reconstruction, the particles should be present in all potential orientations. A limitation in plunge freezing is that the particles interact with the air/water boundary, resulting in preferential orientation and thereby limiting 3D reconstruction. Soft-landing eliminates this issue, but the warming, rehydration could present a similar issue, so preferably the laser power, pulse width, and timing is adjustable in order to control and optimize these parameters and provide preferential orientation of the particles.

**[0121]** Example 8 – Discussion

**[0122]** Previous work has reported on the modification of a hybrid Orbitrap mass spectrometer to permit the landing of protein–protein complex cations onto TEM grids at room temperature. However, in initial experiments the deposition of particles onto bare carbon TEM grids with negative stain TEM analysis showed that the particles had degraded. Recent work confirmed this degradation by imaging room temperature-landed particles,

subsequently plunged into liquid nitrogen and kept cold for imaging (Esser et al., Faraday Discussions 2022, 240: 67-80). The resulting 3D reconstructions of β-galactosidase appear comparable to those shown in **Figure 2** here.

**[0123]** The use of a chemical landing matrix was attempted to address the problem of particle degradation, which allowed for the highest resolution attainable using negative stains (Salome et al., Analytical Chemistry 2022, 94(50): 17616-17624; and Westphall et al., Nature Communications 2022, 13(1): 2276). Unfortunately, this matrix is incompatible with cryo-EM, which would afford a higher resolution.

**[0124]** Accordingly, the present invention utilizes a novel cryogenic landing apparatus that allows for the exploration of the effects of a cryogenic landing surface on the preservation of particles and the formation of thin films of amorphous ice *in vacuo*. With this device, considerable advantages are demonstrated with respect to particle preservation over room temperature deposition. And because the cryogenic landing of the particles presumably captures them in their native gas-phase conformation, this work represents the most direct measurement of the gas-phase structure of protein–protein complex cations as they traverse the mass spectrometer collected to date.

**[0125]** There are several possibilities for why cryo-landing better preserves particle structure and improves the distribution of particle orientations. First, the cryogenic temperature may prevent further dehydration of the protein–protein complex cation while also slowing the rate of deleterious surface interactions. Second, the thin film of ice could be partially serving the protective role of a chemical matrix. Third, although the role ice plays in radiation damage is not currently well understood, it is possible that embedding the particles in the ice present in cryo-landed samples leads to less radiation damage by the electron beam (Xue et al., Frontiers in Chemistry 2022, 10: 889203). Finally, in the case of β-galactosidase, more orientations were observed, presumably due to the reduced particle–carbon surface interactions.

**[0126]** Further structural resolution may be obtained by addressing additional causes of structural degradation. For example, as particles transition through the mass spectrometer, it is possible that dehydration degrades the structural integrity of the particles, precluding higher resolution reconstructions even if the particles are subsequently perfectly preserved on the grid surface. Cooling the surface may also not be sufficient to completely prevent the vacuum exposure and/or the surface itself from inducing structural degradation. Additionally, structural damage may be induced upon deposition due to suboptimal deposition energy or due to the presence of droplets. Finally, it is possible that interactions of the charged

particles with the surface could be causing structural degradation (Crowe et al. Cryobiology 1990, 27(3): 219-231; Ruotolo et al., Curr. Opin. Chem. Biol. 2006, 10(5): 402-408; and Zhou et al., Chem Rev 2018, 118(4): 1691-1741).

**[0127]** To limit dehydration of particles, modifications may be made to how the ions are generated and/or the particles may be frozen while still in the gas phase. The goal of gas-phase freezing is to create amorphous ice-coated particles directly after entry into the mass spectrometer—particles that can land on the cryogenically cooled grid already protected from vacuum and degradation. Alternative chemical matrices and grid surface modifications may be made so to be compatible with the requirements of cryo-EM. For instance, controlling the amount of the chemical matrix allows for the protection of the particles while also limiting the negative effects on cryo-EM imaging. Finally, adjusting the parameters of water and particle co-deposition, i.e., water first, water during, water after, and the use of a water molecular beam versus water vapor, the rate of water deposition, and the temperature of deposition, will further limit potential structural degradation.

**[0128]** In summary, the above examples describe a cryogenic landing apparatus that allows for temperature control of a TEM grid within a mass spectrometer environment, formation of amorphous ice, and a means to reheat the TEM grid (and sample thereon) to allow for rehydration of the sample followed by subsequent cooling and re-formation of an amorphous ice layer on the sample. With this device and associated methods, considerably improved structural resolution is demonstrate, as evidenced by cryo-EM imaging of cryogenically landed protein–protein complex cations. In addition to improving structural quality, this process also provided a much more diverse particle orientation than room temperature landing and allows for the direct coupling of mass spectrometry with cryo-EM.

**[0129]** Having now fully described the present invention in some detail by way of illustration and examples for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

**[0130]** When a group of materials, compositions, components or compounds is disclosed herein, it is understood that all individual members of those groups and all subgroups thereof are disclosed separately. Every formulation or combination of components described or exemplified herein can be used to practice the invention, unless

otherwise stated. Whenever a range is given in the specification, for example, a temperature range, a time range, or a composition range, all intermediate ranges and subranges, as well as all individual values included in the ranges given are intended to be included in the disclosure. Additionally, the end points in a given range are to be included within the range. In the disclosure and the claims, “and/or” means additionally or alternatively. Moreover, any use of a term in the singular also encompasses plural forms.

**[0131]** As used herein, “comprising” is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, "consisting of" excludes any element, step, or ingredient not specified in the claim element. As used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim. Any recitation herein of the term “comprising”, particularly in a description of components of a composition or in a description of elements of a device, is understood to encompass those compositions and methods consisting essentially of and consisting of the recited components or elements.

**[0132]** One of ordinary skill in the art will appreciate that starting materials, device elements, analytical methods, mixtures and combinations of components other than those specifically exemplified can be employed in the practice of the invention without resort to undue experimentation. All art-known functional equivalents, of any such materials and methods are intended to be included in this invention. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Headings are used herein for convenience only.

**[0133]** All publications referred to herein are incorporated herein to the extent not inconsistent herewith. Some references provided herein are incorporated by reference to provide details of additional uses of the invention. All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. References cited herein are incorporated by reference herein in their entirety to indicate the state of the art as of their filing date and it is intended that this information can be employed herein, if needed, to exclude specific embodiments that are in the prior art.

**Claims**

1. A cryo-sample preparation system comprising: a) a sample vacuum chamber; b) a sample holder positioned within the sample vacuum chamber, wherein the sample holder comprises a receiving surface; c) a first temperature control means able to provide a first temperature to the receiving surface of the sample holder, wherein the first temperature is -20°C or less; d) an ion or particle generator comprising a vacuum housing having an internal pressure, wherein the ion or particle generator is able to produce a controllable analyte beam containing charged or uncharged analyte particles; e) a vapor source able to provide vapor to the sample vacuum chamber; and f) a second temperature control means able to provide a second temperature to the receiving surface of the sample holder, wherein the second temperature is greater than the first temperature,

wherein the ion or particle generator is able to direct the analyte beam to contact the receiving surface of the sample holder, thereby depositing analyte particles onto the receiving surface, and

wherein vapor in the sample vacuum chamber is able to generate a solid around, under, and/or over the analyte particles deposited on the receiving surface.

2. The cryo-sample preparation system of claim 1, wherein the vacuum housing comprises a vacuum gate able to isolate the sample vacuum chamber from the ion or particle generator, and the sample vacuum chamber is able to produce an internal pressure different from the internal pressure of the ion or particle generator.

3. The cryo-sample preparation system of claims 1 or 2, wherein the solid is an amorphous solid.

4. The cryo-sample preparation system of claims 1 or 2, wherein a portion of the solid around, under, and/or over the analyte particles is converted to a liquid when the second temperature control means provides the second temperature to the receiving surface of the sample holder.

5. The cryo-sample preparation system of claim 4, wherein the vapor comprises water vapor and the solid layer is an ice layer and wherein the liquid water interacts with the analyte particles to form a rehydrated sample.

6. The cryo-sample preparation system of claim 4, wherein an amorphous solid is formed or re-formed around, under, and/or over the rehydrated sample using the first temperature control means to cool the converted liquid.

7. The cryo-sample preparation system of claim 6, wherein the first temperature control means provides a cooling temperature of -20°C or less to the converted liquid.

8. The cryo-sample preparation system of claim 6, wherein the first temperature control means provides a cooling temperature of -175°C or less to the converted liquid.

9. The cryo-sample preparation system of claims 1 or 2, wherein the vapor is provided to the vacuum chamber as part of the analyte beam containing the charged or uncharged analyte particles, as background gas present in the vacuum chamber as the analyte particles are deposited on the receiving surface, or combinations thereof.

10. The cryo-sample preparation system of any of claims 1-9, wherein the vapor source is able to provide vapor to the sample vacuum chamber prior to the analyte particles being deposited on the receiving surface, concurrently with the analyte particles being deposited on the receiving surface, after the analyte particles are deposited on the receiving surface, or combinations thereof.

11. The cryo-sample preparation system of any of claims 1-10, wherein the vapor comprises molecules or atoms able to form amorphous solids, said molecules or atoms comprising one or more of cyclohexanol, methanol, ethanol, isopentane, water, $O_2$, Si, $SiO_2$, S, C, Ge, Fe, Co, and Bi.

12. The cryo-sample preparation system of any of claims 1-11, wherein the vapor comprises water vapor and the solid is an ice layer.

13. The cryo-sample preparation system of any of claims 1-12, wherein the first temperature control means is able to provide a first temperature of -90°C or less to the receiving surface of the sample holder.

14. The cryo-sample preparation system of any of claims 1-13, wherein the first temperature control means is able to provide a first temperature of -150°C or less to the receiving surface of the sample holder.

15. The cryo-sample preparation system of any of claims 1-14, wherein the first temperature control means is able to provide a first temperature of -175°C or less to the receiving surface of the sample holder.

16. The cryo-sample preparation system of any of claims 1-15, wherein the second temperature control means is able to provide a second temperature of -140°C or more to the receiving surface of the sample holder.

17. The cryo-sample preparation system of any of claims 1-16, wherein the second temperature control means is able to provide a second temperature of -90°C or more to the receiving surface of the sample holder.

18. The cryo-sample preparation system of any of claims 1-15, wherein the second temperature control means is able to provide a second temperature of 0°C or more to the receiving surface of the sample holder.

19. The cryo-sample preparation system of any of claims 1-16, wherein the second temperature is greater than the first temperature by at least 15°C.

20. The cryo-sample preparation system of any of claims 1-19, wherein the receiving surface of the sample holder is a transmission electron microscopy (TEM) grid.

21. The cryo-sample preparation system of any of claims 1-20, wherein the analyte particles are ions generated from a device using electrospray ionization (ESI) or laser desorption.

22. The cryo-sample preparation system of any of claims 1-21, wherein the sample vacuum chamber has a pressure equal to or less than $10^{-4}$ Torr.

23. The cryo-sample preparation system of any of claims 2-22, wherein the ion or particle generator comprises an exit lens or aperture within the vacuum housing, wherein the exit lens or aperture is separated from the sample vacuum chamber by the vacuum gate, and wherein the ion or particle generator is able to direct the analyte beam though exit lens or aperture into the sample vacuum chamber when the vacuum gate is opened.

24. The cryo-sample preparation system of claim 23, wherein the vacuum gate is able to close and maintain the internal pressure of the ion or particle generator when the sample holder is removed from sample vacuum chamber.

25. The cryo-sample preparation system of any of claims 1-23 further comprising a linear actuator able to move the sample holder toward the exit lens or aperture to receive the analyte beam and away from the exit lens or aperture to extract the sample holder.

26. The cryo-sample preparation system of any of claims 1-25, wherein the ion or particle generator comprises a mass analyzer able to measure mass-to-charge ratios of ionized analyte particles, filter ionized analyte particles based on the mass-to-charge ratios of the particles, or combinations thereof.

27. The cryo-sample preparation system of any of claims 1-26, wherein the ion or particle generator or the temperature control means is able to cool the analyte particles in the analyte beam to -20°C or less prior to the analyte particles being contacted with the vapor.

28. The cryo-sample preparation system of any of claims 1-27, wherein the ion or particle generator or the temperature control means is able to cool the analyte particles in the analyte beam to -90°C or less prior to the analyte particles being contacted with the vapor.

29. A method for rehydrating an analyte on a substrate comprising the steps of:

a) forming an analyte solution comprising analyte particles and a solvent;

b) generating an analyte beam from the analyte solution, where the analyte beam comprises charged or uncharged analyte particles and molecules of the solvent;

c) directing the analyte beam towards a receiving surface of a substrate under a first pressure such that the analyte particles and solvent molecules impinge on the receiving surface;

d) forming a first solid layer of the solvent molecules on the receiving surface under a first temperature, wherein the first temperature is -20°C or less, wherein the charged or uncharged analyte particles are embedded within the first solid layer;

e) heating the receiving surface to a second temperature, wherein the second temperature is greater than the first temperature.

30. The method of claim 29, wherein heating the receiving surface to the second temperature causes a portion of the first solid layer to undergo a phase change to a liquid solvent.

31. The method of claim 30, wherein a first portion of the liquid solvent interacts with the charged or uncharged analyte particles to form a rehydrated sample.

32. The method of any of claims 29-31 further comprising:

f) forming an amorphous solid layer from a second portion of the liquid solvent under a third temperature of -20°C or less, wherein the rehydrated sample is embedded in the amorphous solid layer.

33. The method of any of claims 29-32, wherein the first temperature is -150°C or less.

34. The method of any of claims 29-32, wherein the second temperature is -90°C or more.

35. The method of any of claims 29-32, wherein the second temperature is 0°C or more.

36. The method of any of claims 29-32, wherein the third temperature is -150°C or less.

37. The method of any of claims 29-32, wherein the first temperature is -150°C or less, the second temperature is -149°C or more, and the third temperature is -150°C or less.

38. A method of depositing analyte particles on a receiving surface of a sample holder comprising the steps of:

a) positioning the sample holder in a sample vacuum chamber, wherein the sample vacuum chamber has an interior and an internal pressure;

b) forming a controllable analyte beam containing charged or uncharged analyte particles in an ion or particle generator, wherein the ion or particle generator has an interior and an internal pressure and comprises a vacuum gate, wherein the vacuum gate separates the interior of the ion or particle generator from the interior of the sample vacuum chamber;

c) directing the analyte beam to contact the receiving surface of the sample holder, thereby depositing analyte particles onto the receiving surface;

d) reducing the temperature of the receiving surface so that the receiving surface has a first temperature of -20°C or less when contacted by the analyte beam; and

e) providing a vapor to the sample vacuum chamber, wherein the vapor generates an amorphous solid around, under, and/or over the analyte particles deposited on the receiving surface.

39. The method of claim 38 further comprising heating the substrate surface to a second temperature, wherein the second temperature is greater than the first temperature, wherein a portion of the first solid layer undergoes a phase change to a liquid solvent.

40. The method of claim 39, wherein the second temperature is greater than the first temperature by at least 15°C.

41. The method of any of claims 38-39, wherein the receiving surface has a first temperature of -90°C or less when contacted by the analyte beam.

42. The method of any of claims 38-39, wherein the receiving surface has a first temperature of -150°C or less when contacted by the analyte beam.

43. The method of any of claims 38-39, wherein the receiving surface has a first temperature of -175°C or less when contacted by the analyte beam.

44. The method of any of claims 38-43 comprising generating the amorphous solid around the analyte particles prior to the analyte particles being deposited on the receiving surface.

45. The method of any of claims 38-44 comprising generating the amorphous solid around the analyte particles concurrently with the analyte particles being deposited on the receiving surface.

46. The method of any of claims 38-45, wherein forming a controllable analyte beam comprises generating ions using electrospray ionization (ESI) or laser desorption.

47. The method of any of claims 38-46 comprising reducing the internal pressure of the sample vacuum chamber, so that the internal pressure of the sample vacuum chamber is equal to or less than $10^{-4}$ Torr.

48. The method of any of claims 38-47, wherein the internal pressure of the ion or particle generator is $10^{-3}$ Torr or less.

49. The method of any of claims 38-48 comprising cooling the analyte particles in the in analyte beam to -20°C or less prior to the analyte particles being contacted with the vapor.

50. The method of any of claims 38-49 comprising cooling the analyte particles in the in analyte beam to -90°C or less prior to contacting the analyte particles with the vapor.

51. The method of any of claims 38-50 comprising measuring the mass-to-charge ratio of the analyte particles prior to contacting the analyte particles with the receiving surface.

52. The method of any of claims 38-51 comprising filtering the analyte particles prior to contacting the analyte particles with the receiving surface, wherein filtering the analyte particles comprises selecting a portion of the analyte particles having a mass-to-charge ratio within a desired or pre-determined range of mass-to-charge ratios, wherein the analyte particles in the analyte beam consist of the selected portion of analyte particles.

53. The method of any of claims 38-52 comprising separating the interior of the ion or particle generator from the interior of the sample vacuum chamber until the vacuum gate is opened and the analyte beam is directed to contact the receiving surface of the sample holder.

54. The method of any of claims 38-53 comprising closing the vacuum gate and separating the interior of the ion or particle generator from the interior of the sample vacuum chamber while the sample holder is removed from the sample vacuum chamber.

55. A cryo-sample preparation system comprising: a) a sample vacuum chamber; b) a sample holder positioned within the sample vacuum chamber, wherein the sample holder comprises a receiving surface; c) a temperature control means able to provide a temperature of -20°C or less to the receiving surface of the sample holder; d) an ion or particle generator comprising a vacuum housing having an internal pressure and a vacuum gate, wherein the ion or particle generator is able to produce a controllable analyte beam containing charged or uncharged analyte particles, and e) a vapor source able to provide vapor to the sample vacuum chamber,

wherein the ion or particle generator is able to direct the analyte beam to contact the receiving surface of the sample holder, thereby depositing analyte particles onto the receiving surface,

wherein vapor in the sample vacuum chamber is able to generate an amorphous solid around, under, and/or over the analyte particles deposited on the receiving surface, and

wherein the vacuum gate is able to isolate the sample vacuum chamber from the ion or particle generator, and the sample vacuum chamber is able to produce an internal pressure different from the internal pressure of the ion or particle generator.

56. The cryo-sample preparation system of claim 55, wherein the vapor source is able to provide vapor to the sample vacuum chamber prior to the analyte particles being deposited on the receiving surface, concurrently with the analyte particles being deposited on the receiving surface, after the analyte particles are deposited on the receiving surface, or combinations thereof.

57. The cryo-sample preparation system of claims 55 or 56, wherein the temperature control means is able to provide a temperature of -90°C or less to the receiving surface of the sample holder.

58. The cryo-sample preparation system of claims 55 or 56, wherein the temperature control means is able to provide a temperature of -150°C or less to the receiving surface of the sample holder.

59. The cryo-sample preparation system of claims 55 or 56, wherein the temperature control means is able to provide a temperature of -175°C or less to the receiving surface of the sample holder.

60. The cryo-sample preparation system of any of claims 55-59, wherein the receiving surface of the sample holder is a transmission electron microscopy (TEM) grid.

61. The cryo-sample preparation system of any of claims 55-60, wherein the analyte particles are ions generated from a device using electrospray ionization (ESI) or laser desorption.

62. The cryo-sample preparation system of any of claims 55-61, wherein the sample vacuum chamber has a pressure equal to or less than $10^{-4}$ Torr.

63. The cryo-sample preparation system of any of claims 55-62, wherein the vapor is provided to the vacuum chamber as a molecular beam directed at the receiving surface, as part of the analyte beam containing the charged or uncharged analyte particles, as background gas present in the vacuum chamber as the analyte particles are deposited on the receiving surface, or combinations thereof.

64. The cryo-sample preparation system of claim 63, wherein the vapor is provided to the vacuum chamber as part of the analyte beam containing the charged or uncharged analyte particles, as background gas present in the vacuum chamber as the analyte particles are deposited on the receiving surface, or combinations thereof.

65. The cryo-sample preparation system of any of claims 55-64, further comprising a heating source able to increase the temperature of the receiving surface after the analyte particles have been deposited on the receiving surface; after a portion of the amorphous solid has been generated on the receiving surface or around, under, and/or over the analyte particles; or both.

66. The cryo-sample preparation system of claim 65, wherein the heating source able to increase the temperature of the receiving surface by at least 15°C.

67. The cryo-sample preparation system of claim 65, wherein the temperature of the receiving surface remains at a temperature of -75°C or less.

68. The cryo-sample preparation system of any of claims 55-67, wherein the ion or particle generator comprises an exit lens or aperture within the vacuum housing, wherein the exit lens or aperture is separated from the sample vacuum chamber by the vacuum gate, and wherein the ion or particle generator is able to direct the analyte beam though exit lens or aperture into the sample vacuum chamber when the vacuum gate is opened.

69. The cryo-sample preparation system of any of claims 55-68 further comprising a linear actuator able to move the sample holder toward the exit lens or aperture to receive the analyte beam and away from the exit lens or aperture to extract the sample holder.

70. The cryo-sample preparation system of any of claims 55-69, wherein the vacuum gate is able to close and maintain the internal pressure of the ion or particle generator when the sample holder is removed from sample vacuum chamber.

71. The cryo-sample preparation system of any of claims 55-70, wherein the ion or particle generator comprises a mass analyzer able to measure mass-to-charge ratios of ionized analyte particles filter ionized analyte particles based on the mass-to-charge ratios of the particles, or combinations thereof.

72. The cryo-sample preparation system of any of claims 55-71, wherein the ion or particle generator or the temperature control means is able to cool the analyte particles in the analyte beam to -20°C or less prior to the analyte particles being contacted with the vapor.

73. The cryo-sample preparation system of any of claims 55-72, wherein the ion or particle generator or the temperature control means is able to cool the analyte particles in the analyte beam to -90°C or less prior to the analyte particles being contacted with the vapor.

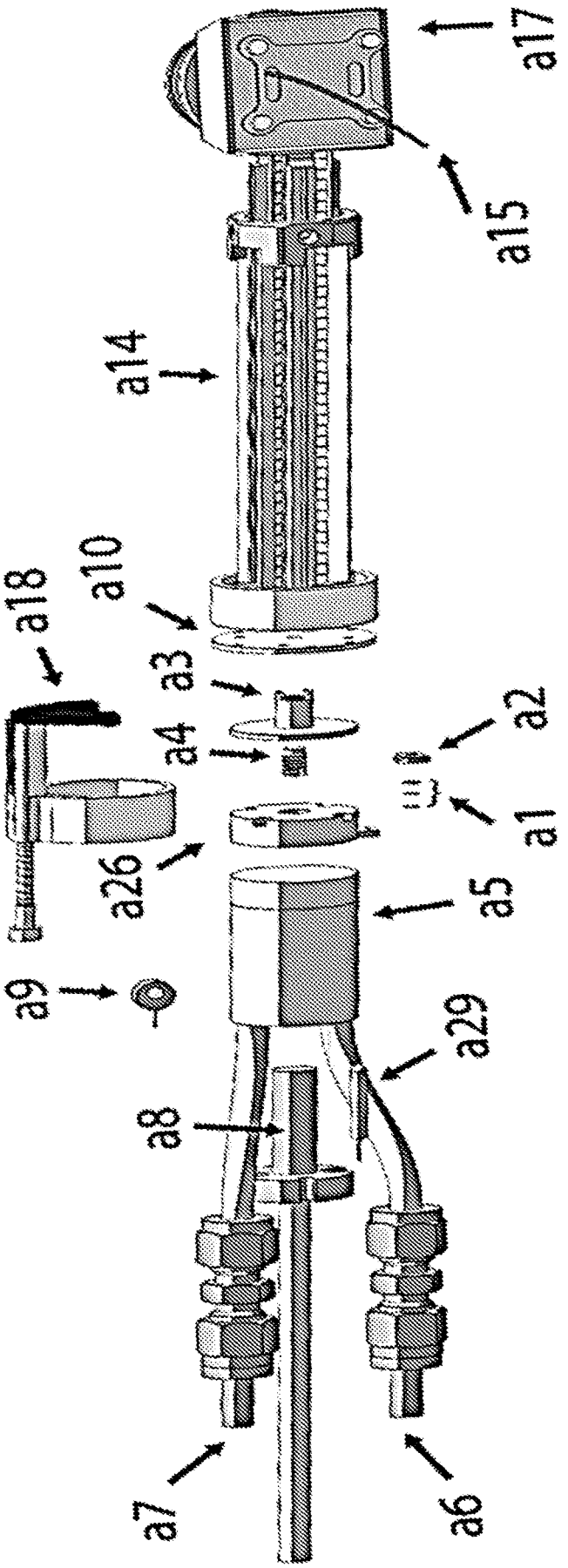


FIG. 1

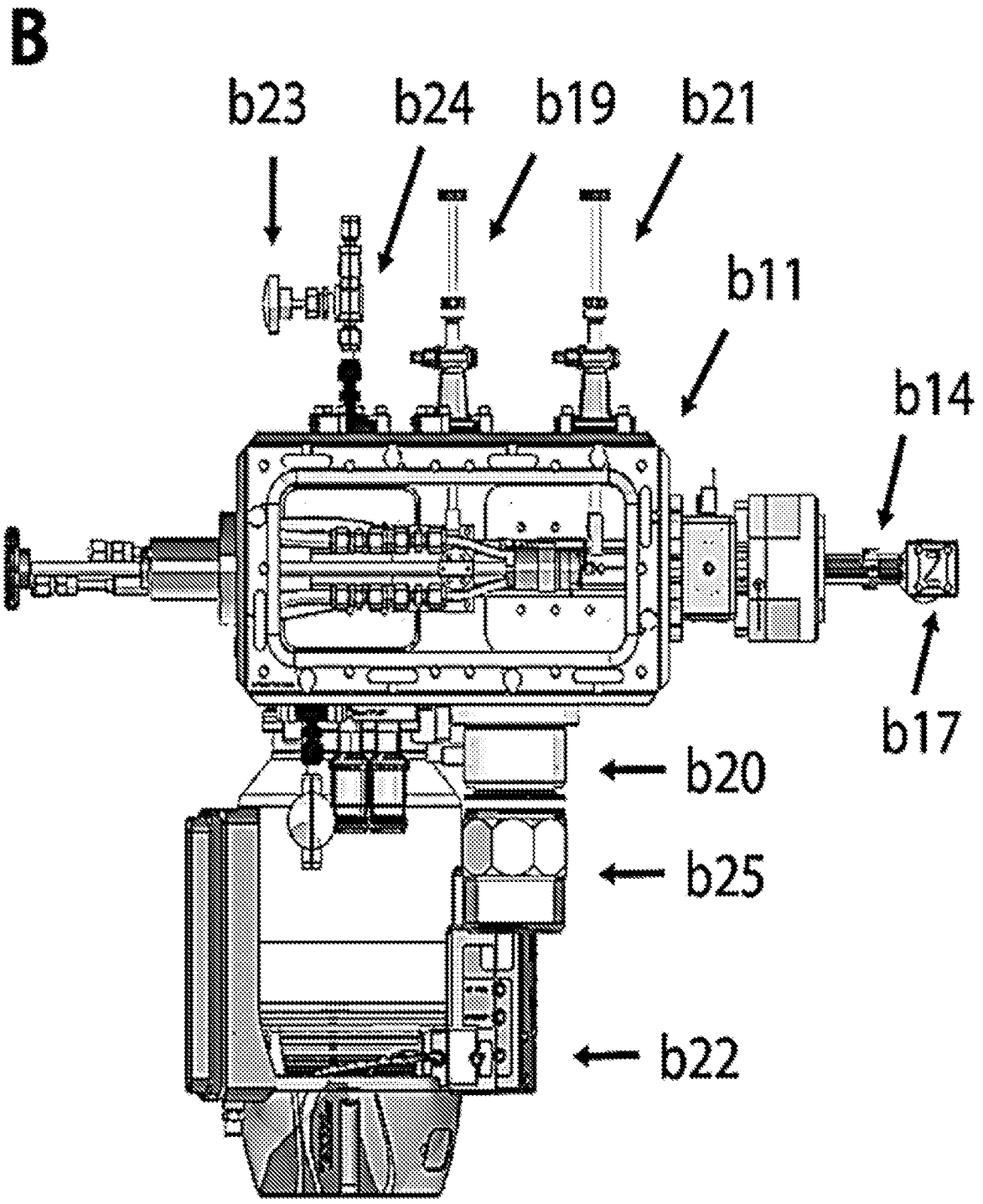


FIG. 1 cont.

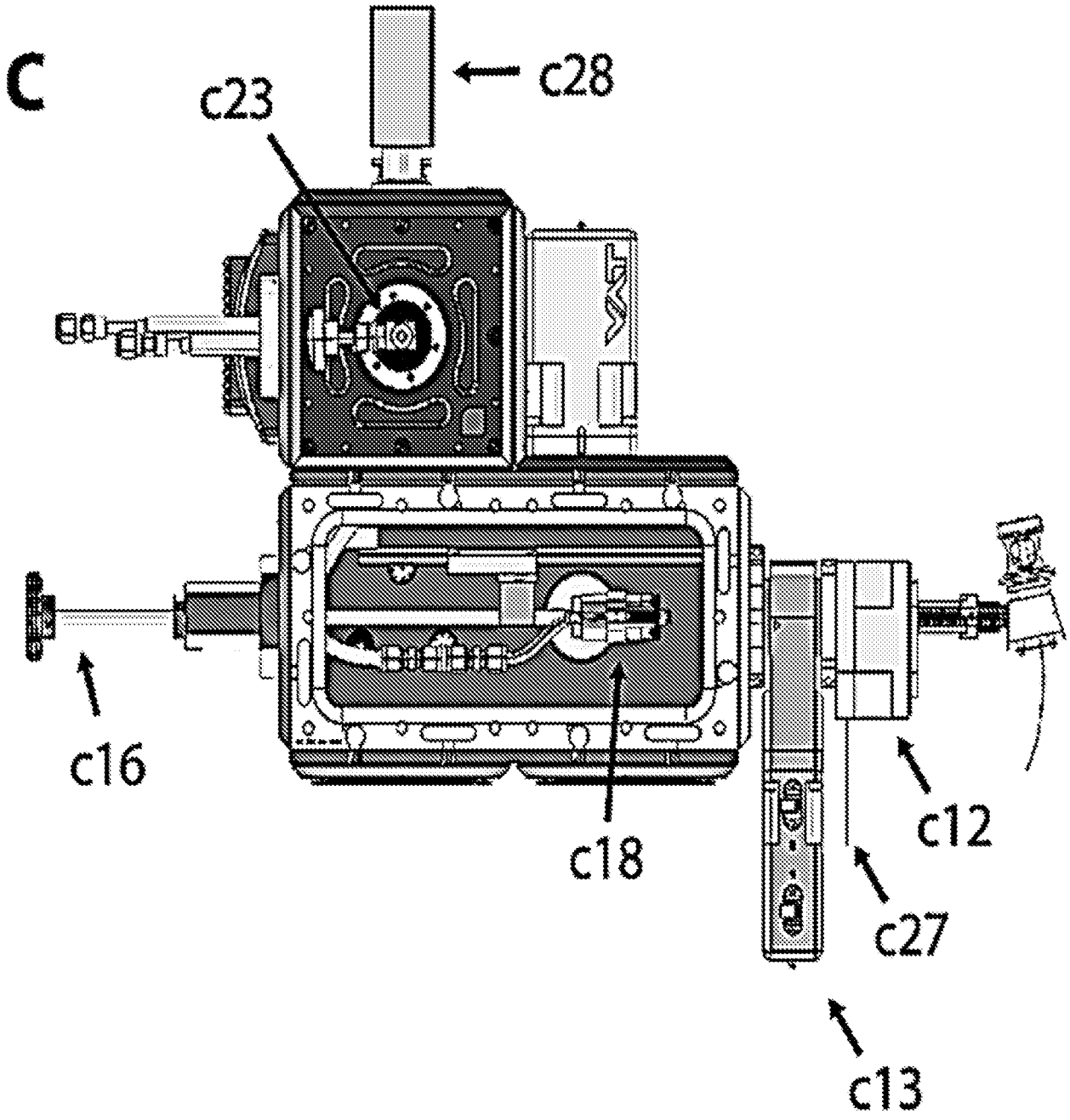


FIG. 1 cont.

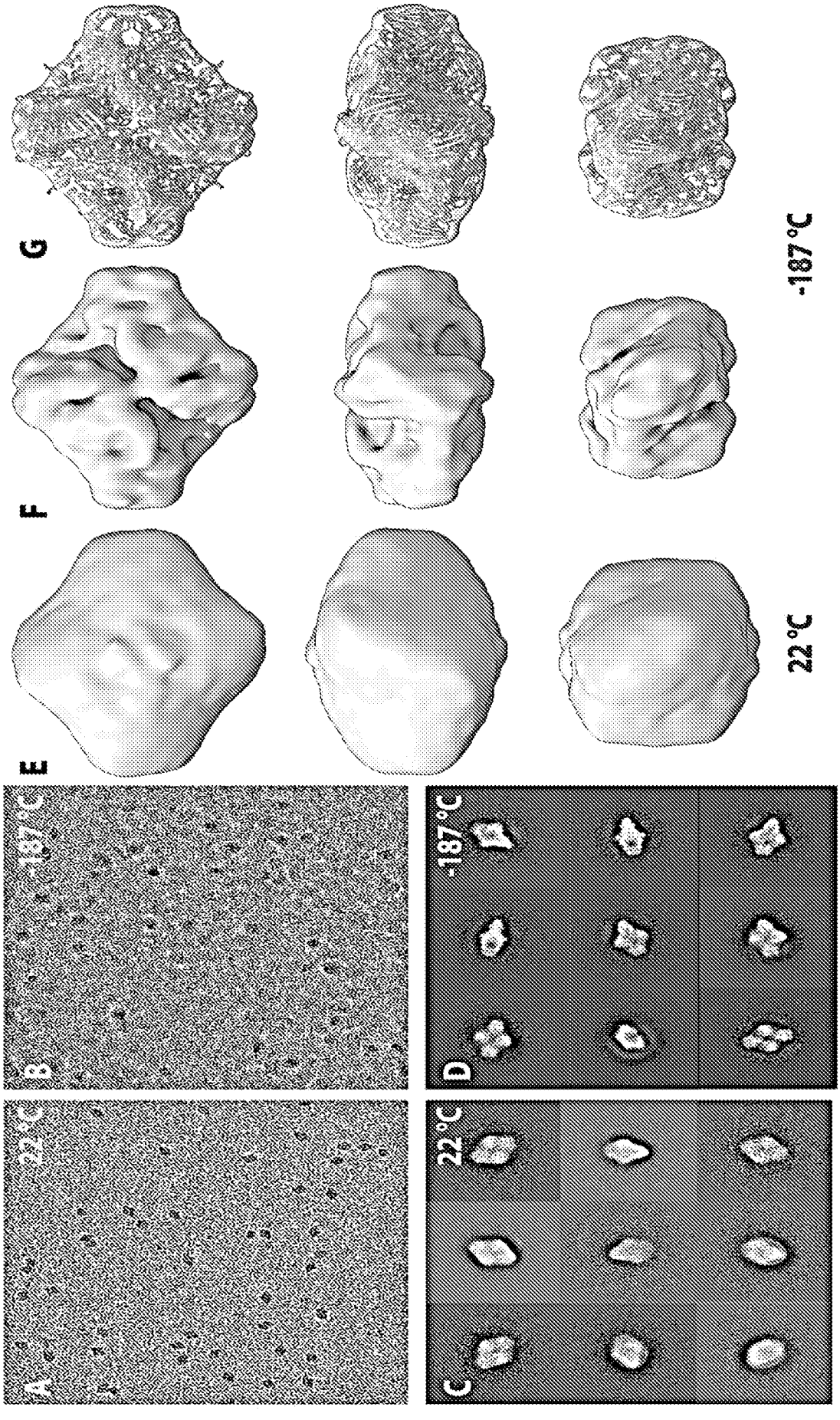


FIG. 2

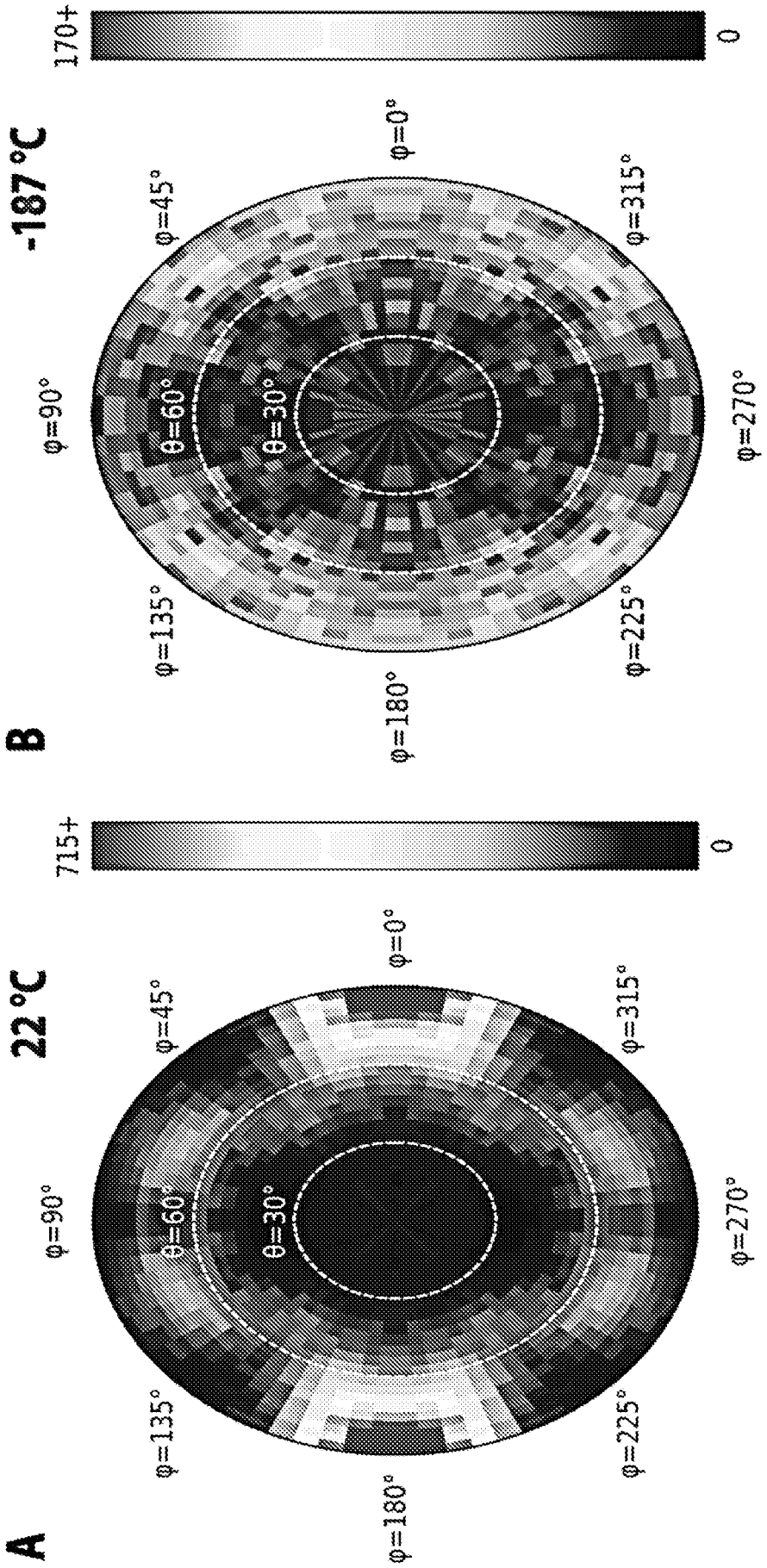
A
22 °C
715+
0
φ=0°
φ=45°
φ=90°
φ=135°
φ=180°
φ=225°
φ=270°
φ=315°
θ=30°
θ=60°
B
-187 °C
170+
0
φ=0°
φ=45°
φ=90°
φ=135°
φ=180°
φ=225°
φ=270°
φ=315°
θ=30°
θ=60°

FIG. 3

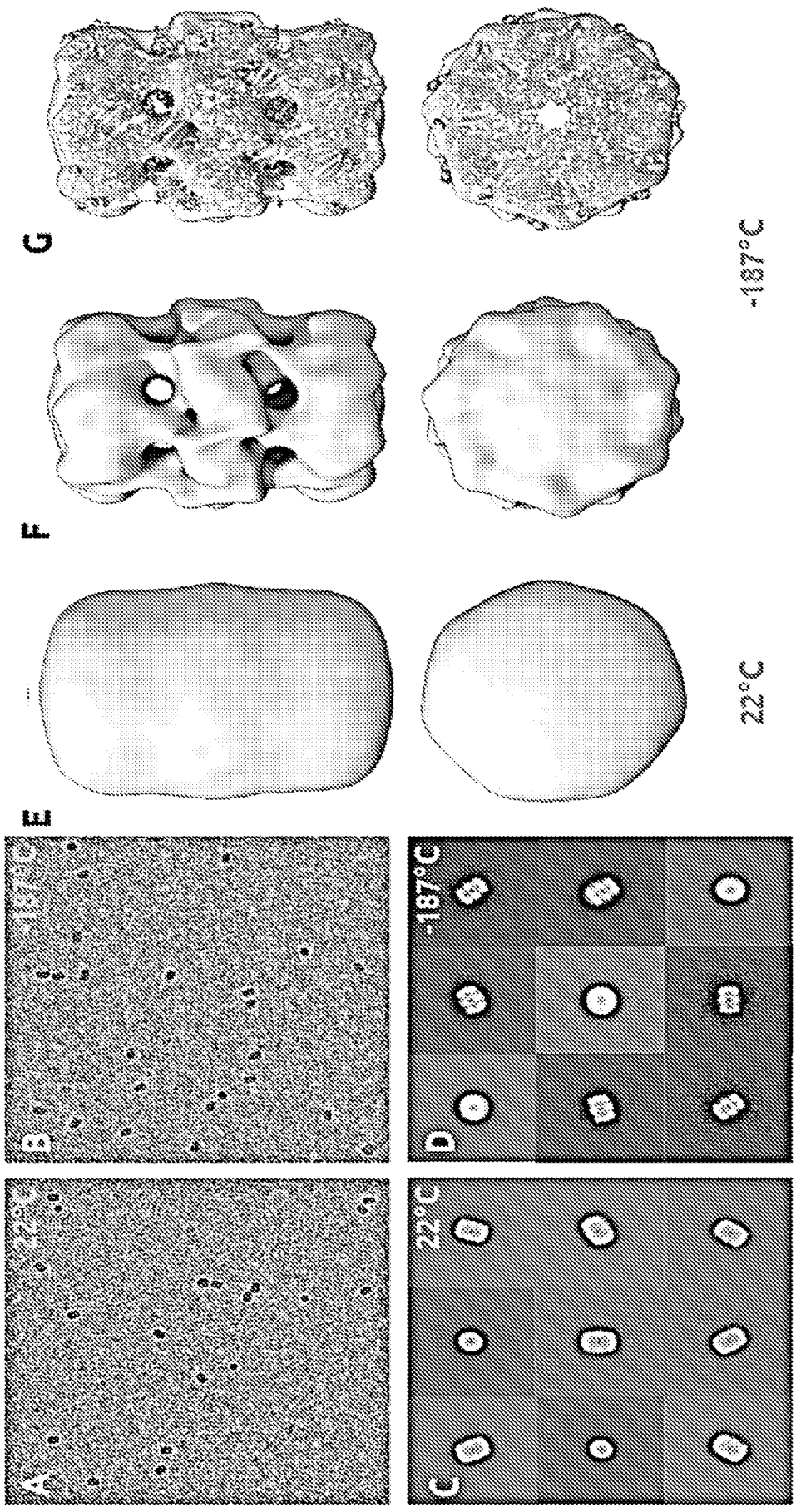


FIG. 4

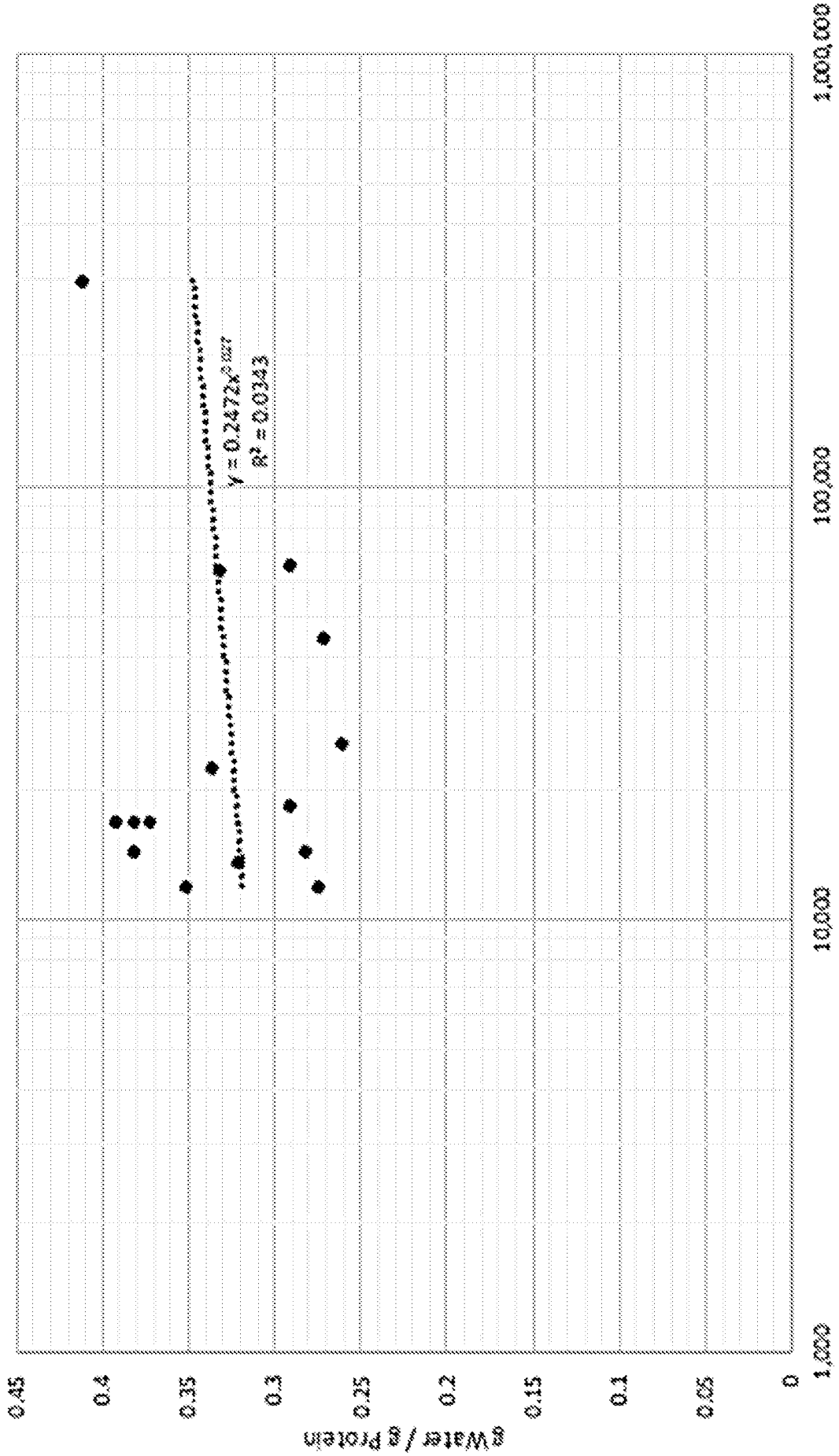
g Water / g Protein
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1,000
10,000
100,000
1,000,000
Protein Molecular Weight (Da)
Literature (Solution)
y = 0.2472x^0.0227
R² = 0.0343

FIG. 5

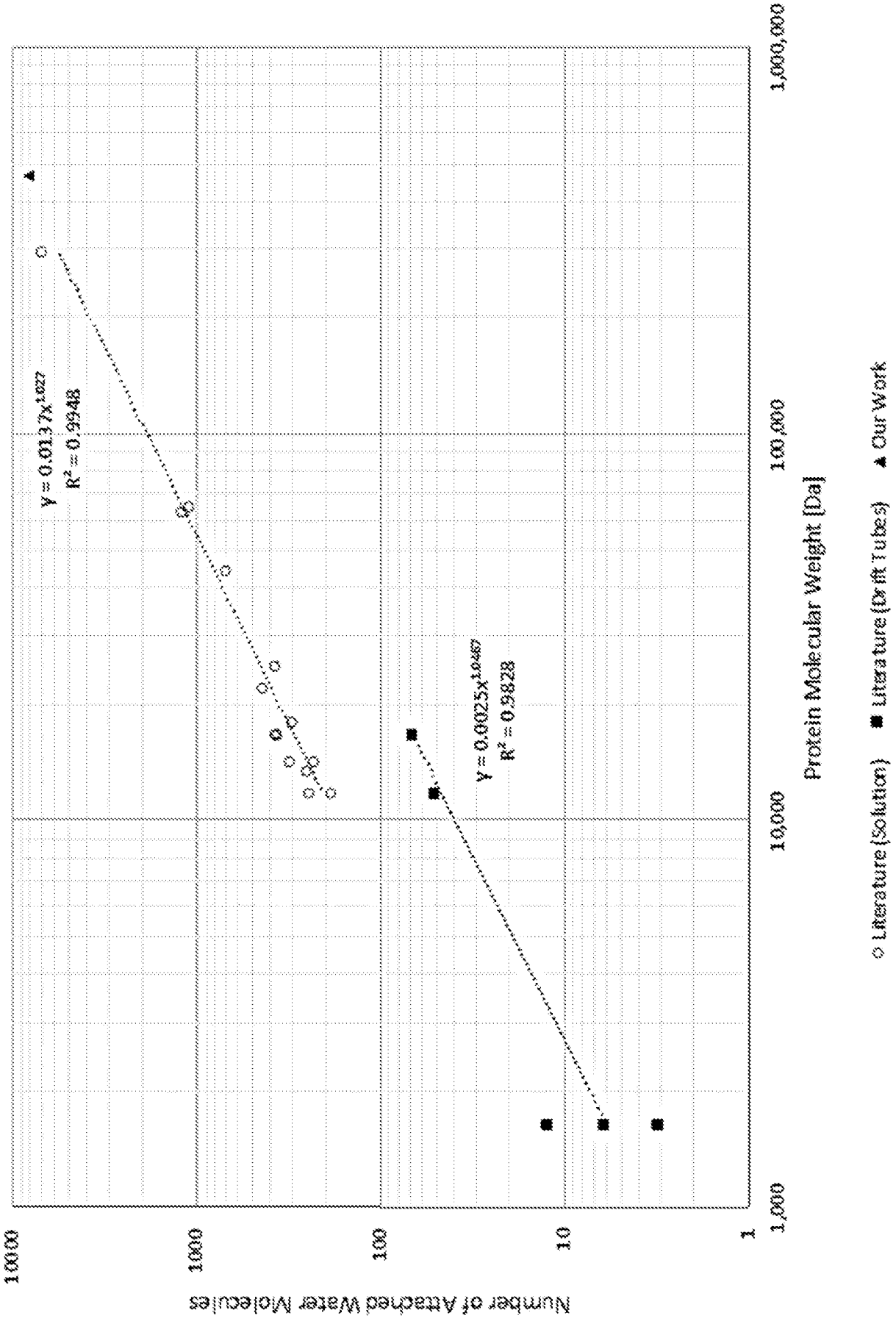
Number of Attached Water Molecules
10000
1000
100
10
1
1,000
10,000
100,000
1,000,000
Protein Molecular Weight [Da]
Y = 0.0137x^1.027
R² = 0.9948
Y = 0.0025x^1.0467
R² = 0.9828
○ Literature (Solution) ■ Literature (Drift Tubes) ▲ Our Work

FIG. 6

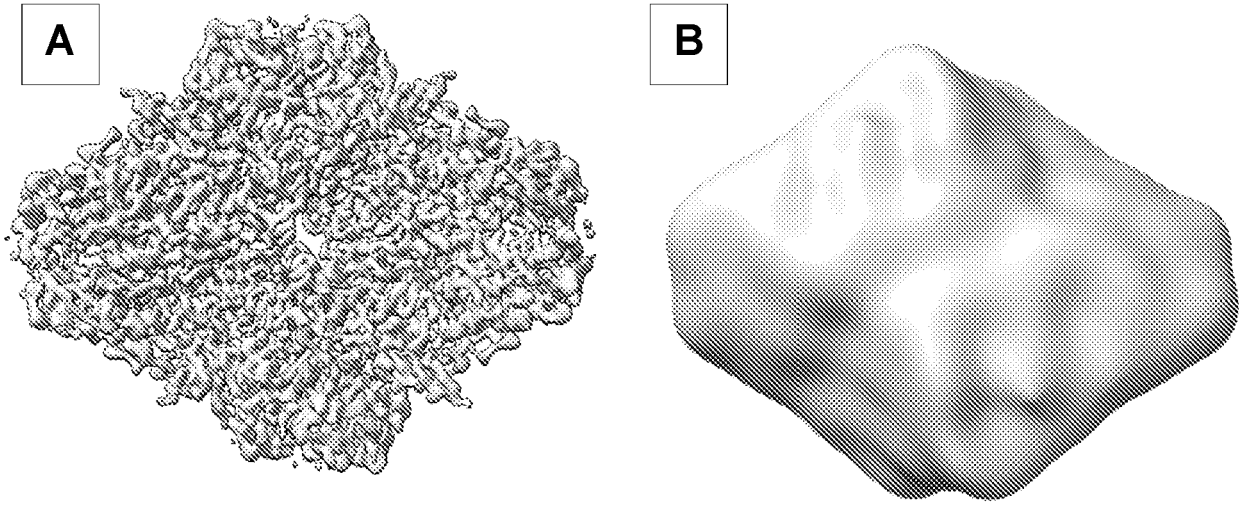


FIG. 7

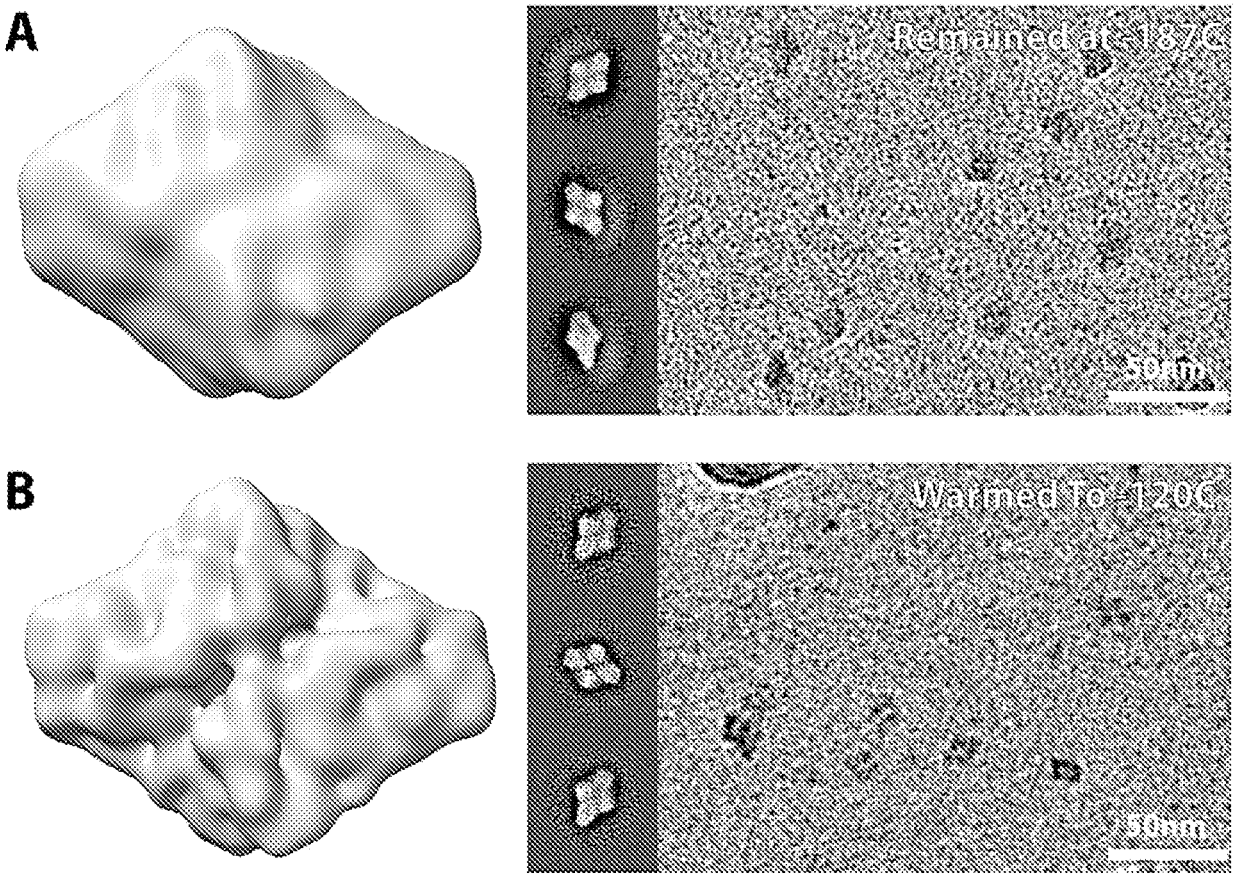


FIG. 8

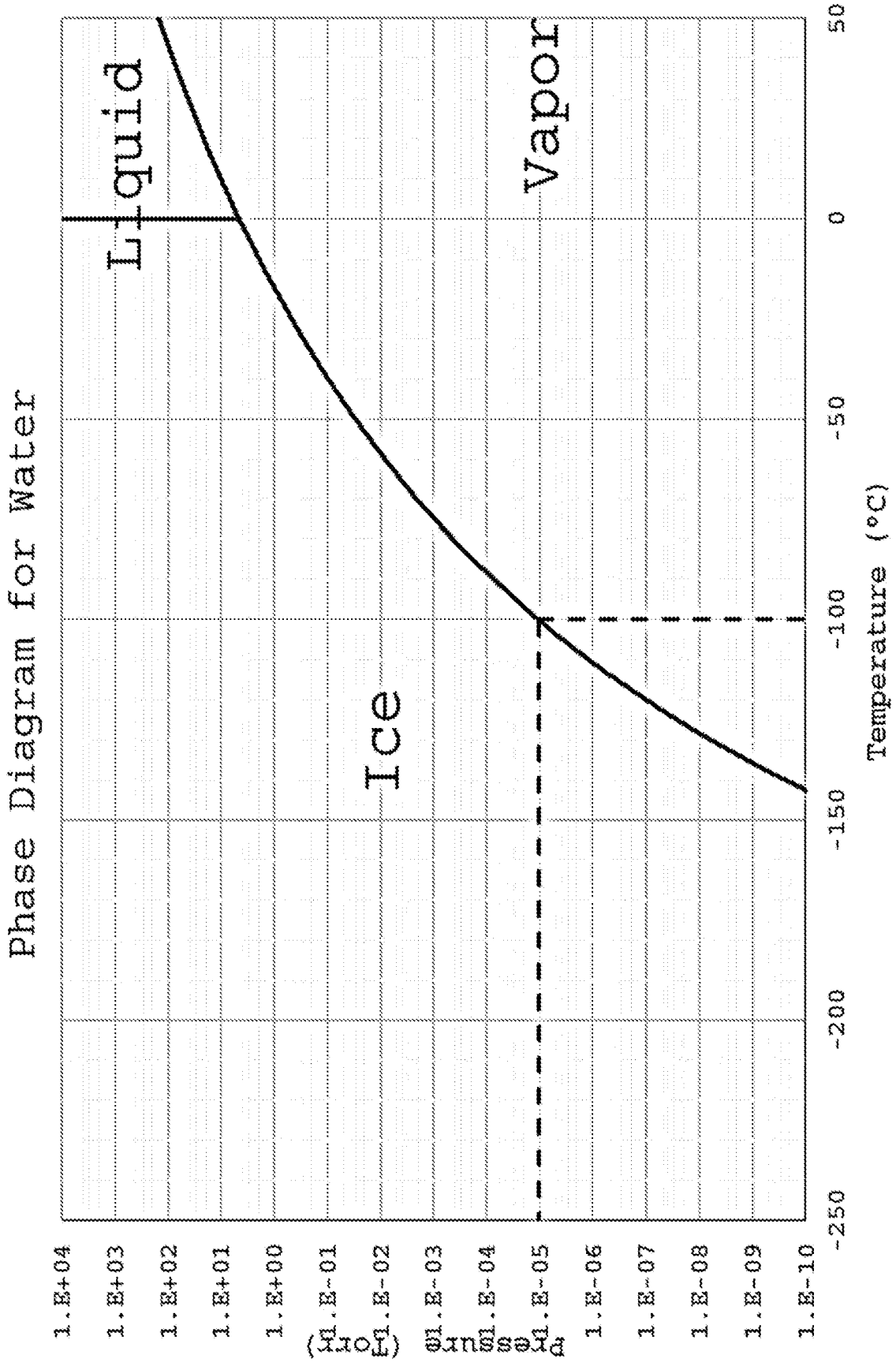
Phase Diagram for Water
Liquid
Vapor
Ice
Pressure (Torr)
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
1.E-05
1.E-06
1.E-07
1.E-08
1.E-09
1.E-10
Temperature (°C)
50
0
-50
-100
-150
-200
-250

FIG. 9

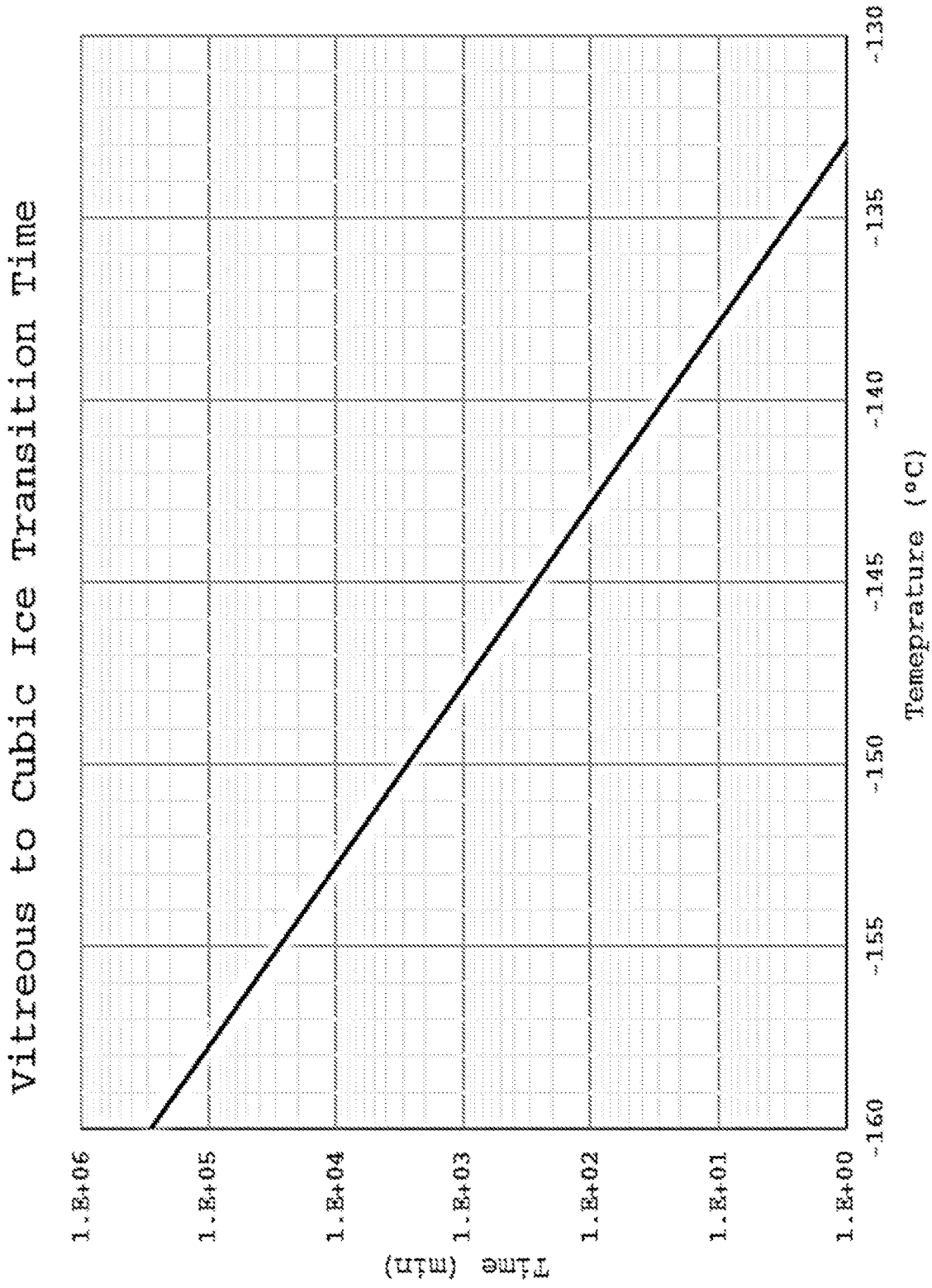
Vitreous to Cubic Ice Transition Time
Time (min)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
-160
-155
-150
-145
-140
-135
-130
Temeprature (°C)

FIG. 10

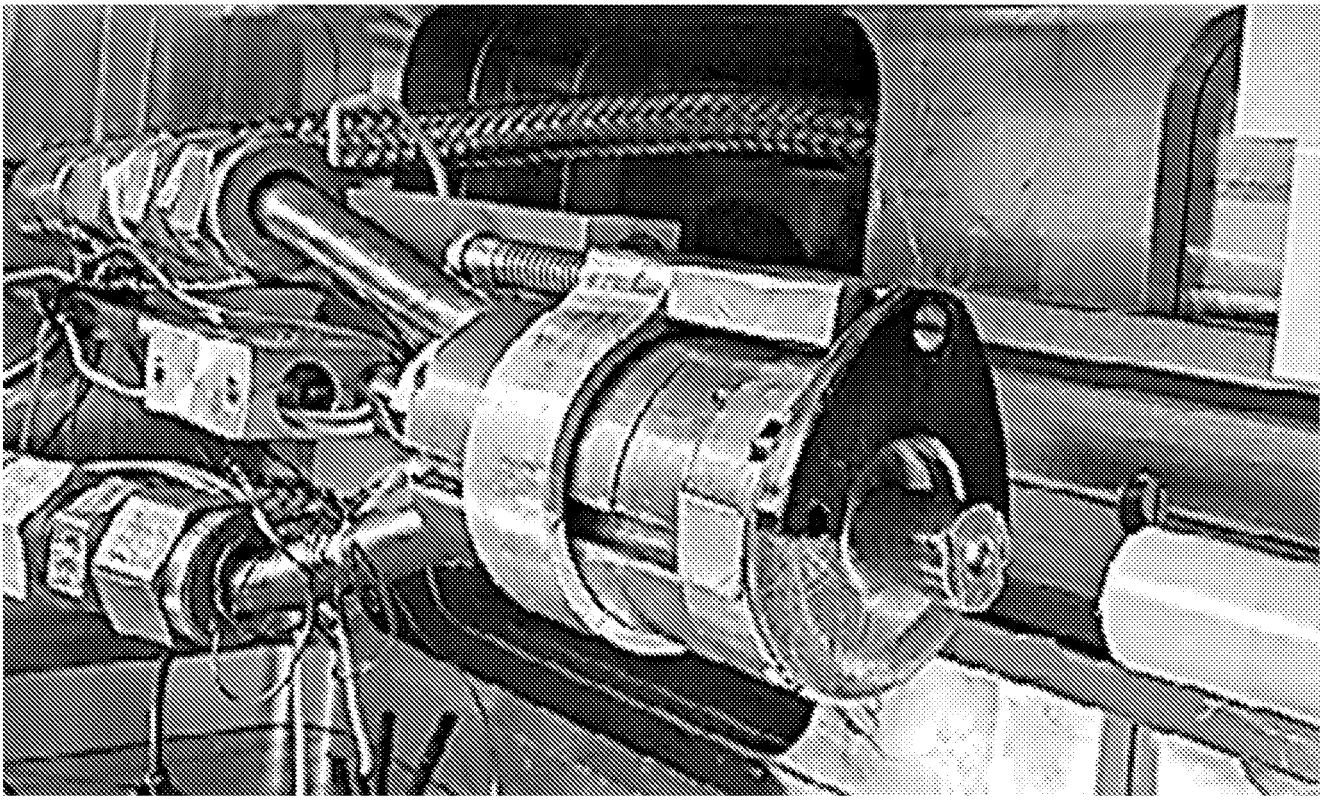

FIG. 11

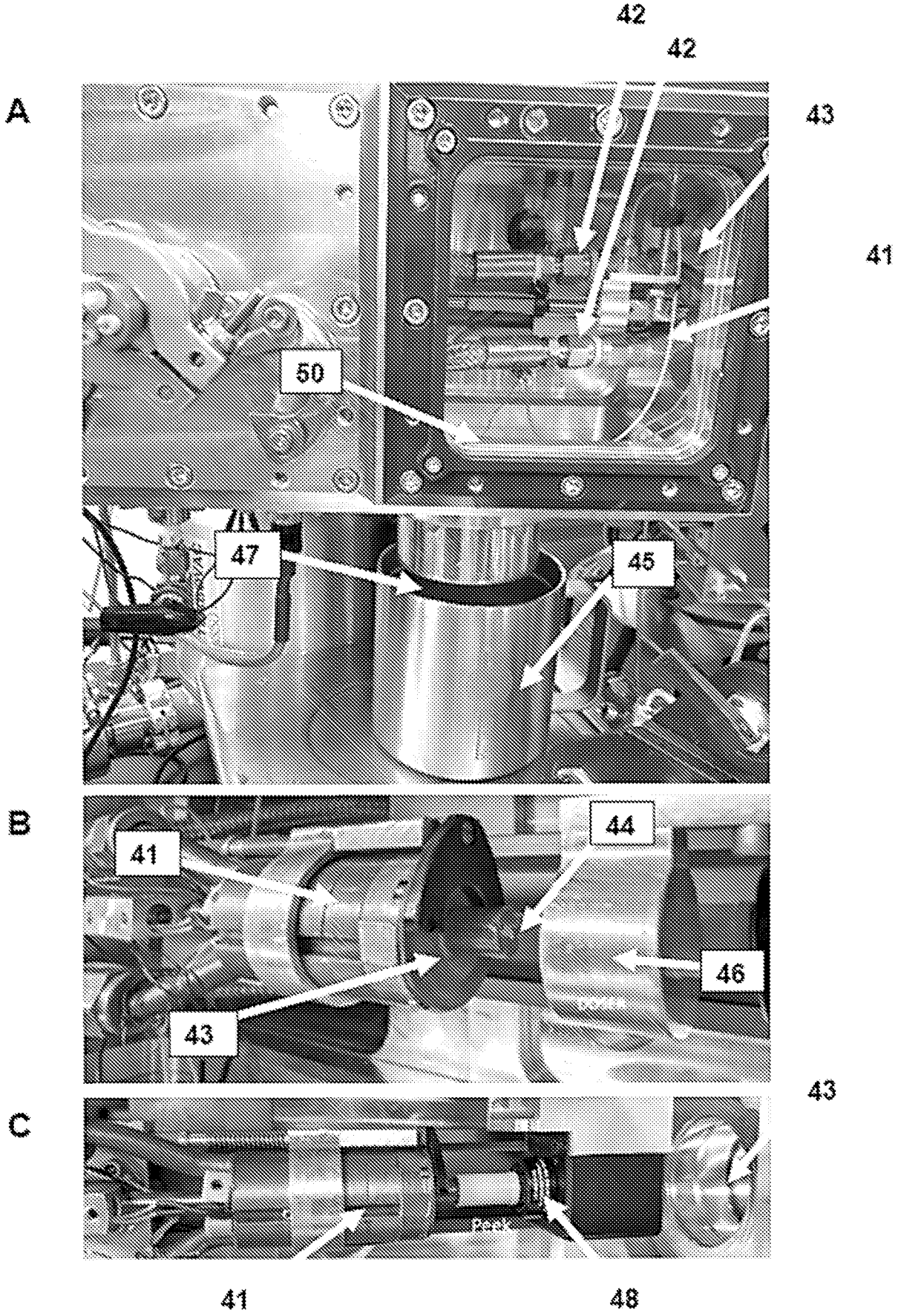


FIG. 12