Published in final edited form as:

J Phys Chem A. 2017 September 14; 121(36): 6671–6678. doi:10.1021/acs.jpca.7b05718.

Doping of Green Fluorescent Protein into Superfluid Helium Droplets: Size and Velocity of Doped Droplets

Maha Alghamdi¹, Jie Zhang¹, Andrew Oswalt¹, Joseph J. Porter², Ryan A. Mehl², and Wei Kong^{1,*}

¹Department of Chemistry, Oregon State University, Corvallis, Oregon 97331, USA

²Department of Biochemistry and Biophysics, Oregon State University, 2011 Agricultural and Life Science building, Corvallis, Oregon 97331, USA

Abstract

We report doping of green fluorescent protein from an electrospray ionization (ESI) source into superfluid helium droplets. From analyses of the time profiles of the doped droplets, we identify two distinct groups of droplets. The faster group has a smaller average size, on the order of 10^6 helium atoms/droplet, and the slower group is much larger, by at least an order of magnitude. The relative populations of these two groups depend on the temperature of the droplet source: from 11 K to 5 K, the signal intensity of the slower droplet group gradually increases, from near the detection limit to comparable to that of the faster group. We postulate that the smaller droplets are formed via condensation of gaseous helium upon expansion from the pulsed valve, while the larger droplets develop from fragmentation of ejected liquid helium. Our results on the size and velocity of the condensation peak at higher source temperatures (> 7 K) agree with previous reports, but those at lower temperatures (< 7 K) seem to be off. We attribute this discrepancy to the masking effect of the exceedingly large droplets from the fragmentation peak in previous measurements of droplet sizes. Within the temperature range of our investigation, although the expansion condition changes from subcritical to supercritical, there is no abrupt change in either the velocity distribution or the size distribution of the condensation peak, and the most salient effect is in the increasing intensity of the fragmentation peak. The absolute doping efficiency, as expressed by the ratio of ion doped droplets over the total number of ions from the ESI source, is on the order of 10^{-4} , while only hundreds of doped ions have been detected. Further improvements in the ESI source are key to extending the technology for future experiments. On the other hand, the separation of the two groups of droplets in velocity is beneficial for size selection of only the smaller droplets for future experiments of electron diffraction.

TOC image

*Corresponding author, wei.kong@oregonstate.edu, 541-737-6714.

Supporting information

Included are figures for the charge distribution of GFP from the ESI source, effect of the bias voltage on the collector/stop electrode on the number of doped droplets, arrival times of Cs^+ doped droplets on the Daly detector, linear regression fittings of the arrival time of the condensation peak of pure droplets, and exit gate opening time of the ESI source relative to the flight times of the condensation and fragmentation peaks.

Introduction

Superfluid helium droplets are ideal coolants for gas phase spectroscopic investigations and for production of exotic species.^{1–6} Recently, we have been engaged in constructing a molecular goniometer by taking advantage of the superfluidity and the ultra-cold temperature of the droplets.^{7–9} At 0.4 K, a moderate laser field is sufficient to achieve a high degree of directional control of an anisotropic species.¹⁰ Diffraction images of molecules all oriented in the desired direction can then be accumulated for determination of the atomic structure of the doped sample. Ultimately, this approach of serial single molecule diffraction has the potential of solving the crystallization problem in crystallography, from nanomaterials to biological molecules.¹¹

A key issue of the molecular goniometer is doping of the target species into superfluid helium droplets. Isolated protein ions can be extracted into vacuum using electrospray ionization (ESI), and the extraction process can be gentle enough to preserve major features of the native conformation.^{12,13} Hence a natural approach for the molecular goniometer is to combine an ESI source with a superfluid helium droplet source.^{14–18} In the report of Bierau et al.,¹⁴ an ion trap with the appropriate buffer gas was used to accumulate a large number of ions, and the buffer gas was pumped down before a pulsed superfluid droplet beam was introduced into the ion trap. The authors reported detection of cytochrome C doped droplets with an average size of 10^{12} helium atoms/droplet. Using a different droplet source but with essentially the same setup, Filsinger et al.¹⁵ reported absorption spectroscopy of hemin⁺ doped in superfluid helium droplets. In addition to a much smaller droplet size, the authors also observed non-thermal ejection of bare hemin⁺. Thereafter, Florez et al. recorded the vibrational spectra of [Leu-Enk + H]⁺ and its complex with crown-16 [Leu-Enk + CE + H]⁺ embedded in helium droplets.¹⁶ Based on the small spectral shift, the authors confirmed the weak interaction between the dopant – albeit charged – with the helium environment. Our group has examined the effects of kinetic energy and droplet size on the doping efficiency using a different approach. By simply slowing down the incident ions in the doping region, nearly 10^4 ions/pulse can be successfully doped in helium droplets.^{17,18} The doping efficiency is discovered to be determined by the ability of a droplet to slow down the incident ion. Hence a less energetic ion and a larger droplet are both beneficial for doping. In

addition, when the duration of a droplet beam is much longer than that of the ion beam, the doping efficiency is proportional to the residence time of the ions in the droplet beam.

In this work, we further extend our method to doping of green fluorescent protein (GFP) into superfluid helium droplets. We have upgraded our cryostat to reach a source temperature of 5 K. This upgrade is essential in generating large droplets for pickup of large dopants, and it allows us to explore the two different formation regimes of superfluid helium droplets, from subcritical to supercritical.¹⁹ We explore the different conditions that affect the doping process, and report the velocity and size distribution of the doped droplets. Although qualitative, we report an absolute doping efficiency of 10^{-4} , and an absolute number of doped ions on the order of several hundreds. The effect of helium atoms surrounding a doped GFP ion on the electron diffraction signal, and the possibility of size selection will also be discussed.

Experimental setup

Figure 1 shows the experimental setup, which is largely similar to our previous reports but with an upgraded cryostat (Sumitomo, SRDK-415D2).^{17,18} The droplet source is a pulsed valve (Parker Hannifin Corp, Series 99) with a nozzle diameter of 500 μm . Ultrapure high pressure helium (99.999%) is cooled in the pulsed valve and forms the superfluid helium droplets upon supersonic expansion. A skimmer, 5 cm in length and 2 mm in diameter, is placed downstream from the pulsed valve. After leaving the skimmer, the droplet beam intersects with the GFP ions coming from the perpendicular direction.

The ion source (Bruker Daltonics, BioTOF) includes an electrospray ionization section, a quadrupole mass spectrometer, and a quadrupole ion trap. During the experiment, the mass spectrometer is set as an ion guide, and all ions from the spray capillary are sent to the ion trap. A pulsed exit gate releases the ions from the ion trap and a custom-made einzel lens focuses the released ion beam into the path of the droplet beam. The width of the ion pulse is determined by the width of the exit gate, which is set at 0.2 ms. Without any external bias, the collector/stop electrode can be connected to an operational amplifier and a resistor to count the total number of ions coming from the ESI source. Alternatively, voltages can be applied on the collector/stop electrode and the einzel lens to maximize the doped signal.

The pulsed mode of operation of the droplet beam and the ion source introduces a timing factor, i. e., in addition to spatial overlap, timing overlap is also crucial for effective doping. Moreover, at different source temperatures, the droplet beam travels at different velocities, hence the timing of the exit gate of the ion source has to be adjusted accordingly. This complication, incidentally, offers additional information on the speed of the droplet beam, as will be discussed in the following section.

Downstream from the droplet source, a retardation electrode is used to determine the size distribution of the doped droplets. The electrode is a metal plate with a mesh covered hole (2.5 cm in diameter) in the middle for droplet transmission, and it can be biased at a positive voltage up to 4000 V. For a group of charged droplets moving at the same velocity, different sizes correspond to different kinetic energies, and only sufficiently large ones can overcome

the positive voltage and go through the mesh for detection. For example, if the velocity of a droplet beam is determined to be 280 m/s, then each helium atom carries a kinetic energy of 1.64 meV. In order to pass through the retardation electrode biased at 200V, a droplet with a +34 charge should contain more than 4.2×10^6 atoms. More details of the size measurement will be provided in the Discussion section.

To probe the doped droplets, further downstream is a Daly dynode detector consisting of a dynode that is biased at -18 kV and an opposing channeltron. The assembly is shielded by a half-cylinder with a 2.5 cm dia. opening to prevent the strong field of the dynode from penetrating into the rest of the chamber. To further prevent bare ions from hitting the dynode detector, a ground mesh is placed in the ion path between the doping region and the Daly dynode. In addition, the einzel lens at the exit of the ESI arm also helps in confining the ions and limiting any leakage of bare ions into the arm of the detector.

In order to calculate the number of ions hitting the dynode, we have placed a copper target further downstream from the Daly detector and wired it to a gigaohm resistor. The Daly detector is a surplus item and neither the material of the dynode nor the gain of the channeltron is known to us. We have solved this problem by performing a calibration experiment using the copper target. From the integrated area of the voltage pulse on the copper target generated by the ion current passing through the resistor, the total number of ion doped droplets can be calculated, and a calibration value for the overall gain of the Daly detector, in the form of a voltage response per doped droplet, can then be established. We have used this method to obtain the absolute number of Cs⁺ doped droplets.¹⁸ Unfortunately, direct calibration using GFP ions could not be accomplished because of the small number of doped droplets. Without efficient amplification, the copper target cannot register any response to the incoming GFP doped droplets. Different from Cs⁺, the GFP ions from our ESI source have a charge distribution from +14 to +70, with a predominant charge of +34 (see Supporting Information, Fig. S1). Hence our estimate of the absolute number of doped droplets obtained from the calibration value of the Cs⁺ experiment can only be considered qualitative.

To facilitate measurements of the velocities of the droplet beam, we have also installed a custom-made fast ion gauge just upstream from the doping region. The ion gauge only consists of a hot filament heated with a current of 1 – 2 Amp and a grid biased at -200 V, but without a collector wire. Hot electrons from the filament are energized by the grid and can ionize the neutral droplet beam. The resulting cations flow with the droplet beam to reach the Daly detector, largely due to the high degree of collimation of the droplet beam, even after ionization and fragmentation. After a long drift distance of ~30 cm, we can observe hundreds of millivolts of signal on the channeltron of the Daly detector. Compared with a collector wire of a typical ion gauge, the Daly detector has a faster response time – on the order of sub-microseconds, which is beneficial to the precision of the velocity measurement. Moreover, the same detector is used for pure droplets and ion doped droplets, offering consistency in the resulting time profiles.

The protein sample, super-folder green fluorescent protein (GFP), was originally engineered for stability and fast maturation by Pedelacq et al.²⁰ It contains 238 amino acid residues with

a molecular weight of 28 kDa. Its tertiary structure is a cylinder of 2.5 nm in diameter and 4.0 nm in height. Eleven β strands form the exterior wall and the chromophore is anchored inside the β barrel.²¹ The current sample was further modified by genetic code expansion to contain para-bromophenylalanine (BrPhe).^{22–24} This site-specific incorporation of a non-canonical amino acid BrPhe into site 150 was to enable future experiments with laser alignment and photofragment imaging. The sample from the Unnatural Protein Facility at Oregon State University at 20 mM was dissolved in 1:1 water to acetonitrile with 0.1% formic acid to achieve a sample concentration of 20 μ M. The expected mass of the resulting super-folder GFP-BrPhe is 27939.3 ± 1 Da. The predominant peak of the mass spectrum was at 822 atomic units (amu, mass-to-charge ratio), corresponding to a charge of +34 (see Supporting Information, Fig. S1). The experiment was repeated using the same method but without any formic acid. The resulting number of ions was similar, but the charge distribution shifted to lower values, with a predominant mass-to-charge ratio of 1863 amu, corresponding to a charge of +15. However, clogging of the capillary in the ESI source became an issue without formic acid; hence all results reported hereafter were obtained with formic acid. At a flow rate of 650 μ L/h, the number of ions was $\sim 10^6$ ion/pulse on the collector/stop electrode.

Results

Figure 2 shows the intensities and arrival times of GFP doped droplets measured on the Daly detector at different temperatures of the droplet source. The origin of the abscissa is the time of the electrical trigger to the pulsed valve. The relative timing of the exit gate of the ion source was also adjusted at each temperature of the droplet source to maximize the overall number of ion doped droplets on the Daly detector, and this information is presented in Fig. S5. The stagnation pressure was 26 atm and the duration of the trigger pulse to the pulsed valve was 85 μ s. The bias voltage on the collector/stop electrode was 34 V. Similar to our previous work on reserpine and substance P,¹⁷ the doping efficiency depends on the bias of the collector/stop electrode (see Supporting Information, Fig. S2): it shows an abrupt increase at ~ 20 V, maximizes between 30 – 45 V, and then drops sharply above 45 V. At the optimal bias value of 34 V, the number of doped droplets is tripled compared with no bias on the electrode.

In Fig. 2, the intensity of the GFP doped droplet first increases with decreasing source temperature, from 11 K to a maximum at 7 K, and then slightly decreases between 7 K and 5 K. Each trace contains up to two major peaks and one minor peak in between, but the relative strength of the slowest peak in each trace increases with decreasing temperature. For reasons which will be justified in the Discussion section, we name the fastest peak the “condensation peak”, the middle peak the “rebound peak”, and the slowest peak the “fragmentation peak”. The onset of all peaks shifts to a later time with decreasing temperature; the time shift from 11 K to 5 K is 0.41 ms. Similar to all pulsed droplet sources,² we also have some instabilities in the experimental results, but the general trend of intensity change is repeatable under all conditions.

The time-of-flight (TOF) profile in Fig. 2 is representative of the time profile of the droplet beam. We have recorded almost identical profiles for Cs⁺ ion doped droplets (see Supporting

Information, Fig. S3) generated from a thermionic source, and similar profiles from ionized pure superfluid helium droplets. In the latter case, ionization was achieved using the hot filament of the custom-made fast ionization gauge (Fig. 1). In addition, the profiles were also observable from the copper target, although due to the slow time response of the electronics system, the rebound pulse was no longer observable, and the fragmentation peak was only a shoulder on the tail of the condensation peak.

Compared with our previous results using the same pulsed droplet source but at different source temperatures,^{17,18} Fig. 2 shows several major differences. The highest source temperature to observe any GFP doped droplets is 11 K, which is below the lowest temperature we could achieve prior to this upgrade (13 K). This is expected since the heat capacity of GFP should be considerably larger than that of Cs⁺. A much larger droplet is therefore required to cool the GFP ions, hence a much lower temperature is required for the droplet source. The number of ion doped droplets reaches a maximum at 7 K, signifying the existence of an optimal condition for the number of doped droplets. The time profiles of the doped droplets at these low temperatures are essentially bimodal, with a clear separation between the two major groups of droplets.

To measure the size distribution of the GFP doped droplets, we have relied on the retardation experiment.^{17,18} During the experiment, we discovered that only the condensation peak and the rebound peak in the TOF profile of the doped droplets decreased with increasing retardation voltage, while the intensity of the fragmentation peak was only marginally affected by the retardation voltage, even at 4000 V. We defined a cutoff timing for the condensation peak using the minimum between this peak and the rebound peak, and integrated the profile to represent the number of ion doped droplets. Although the profiles of the peaks are not quite Gaussian-like, we also tried to fit the two major peaks using Gaussian profiles for simplicity. The intensity distributions of the condensation peak from the fitting of the Gaussian profiles are shown in Fig. 3a, together with that of the fragmentation peak at 7 K. Regardless of the treatment method, the basic profiles of Fig. 3a remain the same within our error limit (10%).

Measurements from the retardation experiment determine the number of ions with sizes above the corresponding cutoff limit at a certain retardation voltage, not the actual size distribution. To derive the size distribution, we need to convert the retardation voltage into droplet sizes and take the derivative of the resulting profile. The conversion from voltage to size requires knowledge of the velocity of the droplet beam, which will be discussed in the next section. After the size conversion, we fitted the experimental data from the condensation peak using a single exponential function

$$y=y_0+Ae^{-\frac{x}{a}}, \quad (1)$$

and the resulting parameters are listed in Table 1. The coefficients of determination for all fittings are also listed for reference. The values of y_0/A are less than 10% at all temperatures, hence within the error limit, it is essentially negligible. We therefore conclude that there are no exceedingly large droplets within the condensation peak.

The differentiation of an exponential function remains an exponential function, and the normalized size distributions at different source temperatures are shown in Fig. 3b. The abscissa starts at 10^5 atoms/droplet to emphasize the variation in the resulting profiles, since on the semi-log scale with a decay constant on the order of 10^6 , the profiles all plateau from 10^5 to size one (1). Although for a continuous nozzle between 11 K and 5 K, the expansion process of the droplet beam should change from subcritical to supercritical,¹⁹ and the size distribution should also change from log-normal to single exponential, within the range of detection, all observed size distributions of GFP doped droplets fit single exponential functions. This is similar to our previous studies of reserpine and substance P,¹⁷ where even at a nozzle temperature of 14 K, the distribution of doped droplets can be modeled with a single exponential decay function. We have attributed this phenomenon to an artifact of the detection range, which results in insensitivity of the large-sized droplets to the mathematical fitting function.^{17,18} On the other hand, regardless of the actual functional form of the size distribution of the pre-doping droplets, the goal of the fitting is to obtain the average size of the doped droplets, and a high quality fitting as shown in Fig. 3 lends credence to the conclusion.

We emphasize here that the size distribution of the ion doped droplet beam might not be reflective of the overall size distribution of the droplet beam prior to doping. In particular, the size of doped droplets does not include the amount of atoms evaporated to stop and cool the GFP ions to the temperature of the droplet. The classical thermal energy of 1000 atoms is about 40 eV, requiring evaporation of 10^4 helium atoms to cool from 300 K to 0.4 K. If the kinetic energy of a GFP ion with a charge of +34 is reduced from 680 eV (gained from a 20 V potential energy) to 100 eV by the collector/stop electrode, the removal of a kinetic energy of 100 eV requires 10^5 helium atoms.

Discussion

Velocities of the droplet beam

We believe that the two major peaks in Fig. 2 originate from different mechanisms of droplet formation instead of rebound of the pulsed valve. Several groups have reported the observation of rebound pulses from Even-Lavie (EL) pulsed valves (Digital Technology Trading & Marketing Ltd., Cryogenic Copper Even-Lavie Valve).^{25–27} Typically a rebound pulse is produced after the primary pulse. Due to fast gas release during the primary pulse, the temperature of helium during rebound is expected to be slightly higher than that during the primary pulse. Consequently, the resulting droplets from a rebound pulse are typically smaller than or comparable to those from the primary pulse, and may travel at a slightly higher speed. In our previous report on the rebound pulse of an EL valve,²⁵ we did observe that the droplets from the rebound pulse were similar to or smaller than that of the primary pulse. When the source temperature was at 18 K, the rebound peak even overtook the primary peak and arrived at the detector prior to the primary peak. At a source temperature of 8 K, on the other hand, the rebound peak fell far behind the primary peak, by almost 0.6 ms. The middle small peak in Fig. 2 shows a delay of 0.2 – 0.4 ms relative to the condensation peak but is ahead of the fragmentation peak, in a reasonable range for a

rebound peak. Moreover, it mimics the behavior of the condensation peak in a retardation field, which further corroborates its close association to the condensation peak.

The slowest fragmentation peak, however, cannot be attributed to rebound of the pulsed valve. It exhibits much weaker dependence on the retardation field than the condensation peak, implying a much larger size. Moreover, the fragmentation peak increases in intensity with decreasing source temperatures, and at 7 K, it is as strong as the condensation peak. If the fragmentation peak were truly a strong rebound of the primary condensation peak at 7 K, we would expect more, albeit weaker, rebound peaks after this peak, since the damping force of the pulsed valve must be vanishingly small to produce such a strong rebound pulse. In Fig. 2, the tail of the fragmentation peak shows no oscillatory behavior, hence no further rebounds are detected.

Based on the analysis of Buchenau et al.,¹⁹ the conditions of our experiment belong to regime II and III where the expansion isentropically approaches the liquid-gas phase line from the liquid phase side (regime III) or near the critical point (regime II). Upon reaching the binodal line, the system breaks into two separate phases and the two phases expand alternately into vacuum. While the liquid phase breaks down into droplets during the expansion, the vapor phase condenses into droplets. The condensation process, however, provides additional enthalpy for acceleration, and consequently, droplets formed from the condensation process travel at a higher speed. Although our experiment employs a pulsed valve instead of a continuous nozzle, and the specific criteria for the different regimes might differ,¹⁹ the general physics should be the same. The gradual appearance of the fragmentation peak in Fig. 2 is actually quite similar to the observation of Buchenau et al.¹⁹ Consequently, we believe that the fastest droplet peak should be the condensation peak, and the slowest peak should be the fragmentation peak.

To derive the velocity distribution of the droplet beam under different temperatures, we first used the information from pure droplets to derive the response time of the pulsed valve t_0 (including the response time of the detector) and the distance between the nozzle and the Daly detector d . For expansion conditions above 13 K,¹⁹ the formation mechanism of the droplets should be exclusively gas condensation. In the isentropic expansion, the final velocity v_c should be related to the initial enthalpy $5/2k_B T$ via:²⁸

$$v_c = \sqrt{\frac{5k_B T}{m_{He}}}, \quad (2)$$

where k_B is the Boltzmann constant, T is the temperature of the source, and m_{He} is the mass of a helium atom. The flight time t can then be related to the source temperature via:

$$t = t_0 + \frac{d}{\sqrt{5k_B/m_{He}}} \cdot \frac{1}{\sqrt{T}}. \quad (3)$$

From the arrival time of the pure droplets on the Daly detector, we obtained a response time of 0.23 ms, a distance of 70 cm for the flight region, and a coefficient of determination of 0.97, as shown in Fig. S4 in the Supporting Information. The distance is in agreement with our physical measurement, and the response time is also consistent with the time delay between the electrical pulse and the gas plume from the mechanical movement of the pulsed valve. The response time of the pulsed valve could change with temperature, but given the relatively long flight time (> 2 ms) and the relatively short response time (~ 0.2 ms), this variation could be ignored within the uncertainty of the experiment.

From the arrival times of the doped droplets, the response time of the pulsed valve and the distance of travel, velocities of different groups of ions can be calculated. Fig. 4 shows the results from the data presented in Fig. 2. The uncertainty in the experimental values is about 10%. The theoretical value based on Eq. 2 is also plotted for comparison. In general, the velocity of the fragmentation peak is consistently $\sim 15\%$ lower than that of the corresponding condensation peak. The theoretical values are in general agreement with those of the condensation peak in the temperature region above 7 K, but below 7 K, the theoretical values are substantially lower. Although an energized coil of the pulsed valve could raise the temperature of helium, the rate of heat transfer from the coil to the gas reservoir limits this effect to less than 1%, which is insufficient to explain the discrepancy below 7 K. On the other hand, Eq. 2 is only applicable for subcritical expansion conditions, hence a deviation under 7 K is actually expected. What is surprising is how well the theoretical values agree with the experimental data since the enthalpy in Eq. 2 does not include the condensation enthalpy, hence Eq. 2 should represent a lower limit to the final velocity of the condensation peak. The agreement seems to imply that little if any of the condensation enthalpy is turned into translational energy of the resulting droplet beam.

The calculation for the fragmentation peak depends on the details of the nozzle and expansion conditions. Buchenau et al.¹⁹ have calculated the range of the expected values for a continuous nozzle at pressures between 8 atm and 20 atm. Although the pressure of our pulsed nozzle is at 26 atm, our values for both the condensation peak and the fragmentation peak are in agreement with their calculation at 20 atm in the same temperature range.

Another piece of supportive information of the current values of velocities comes from the timing of the exit gate of the ion source, which is confirmed from Fig. S5 in the Supporting Information. The width of the ion pulse from the exit gate is about 0.2 ms, determined by the temporal width of the exit gate. For maximum doping of both the fragmentation and the condensation peaks, the timing of the exit gate of the ions should be centered between the two pulses.

It is worth noting that the velocity of the droplet beam is dependent on the stagnation pressure: in the work of Grisenti and Toennies,²⁹ at a stagnation pressure of 0.5 atm and a source temperature of 1.6 K, a velocity as low as 15 m/s has been reported. At 30 atm, however, the velocity has increased to nearly 200 m/s. Furthermore, the velocity spread of each peak from our measurement, as seen from Fig. 2, is also on par with those from Grisenti and Toennies²⁹ under comparable conditions.

Size distribution

The fundamental hypothesis of size measurement through retardation is uniform velocity for droplets with different sizes. The TOF profiles of GFP doped droplets in Fig. 2 contain two major peaks corresponding to two groups of droplets with two different velocities. If we treat each group separately, the assumption of a uniform speed for each group of droplets is still valid. The width of each peak, ~10% of the overall flight time, thus represents the velocity spread of each group of droplets, convoluted with the time response of the detector.

The size distribution from the condensation peak is in general agreement with our own previous work^{17,18} and those from Filsinger et al.,¹⁵ but it is very different from that of Bierau et al.¹⁴ On the other hand, the exceedingly large size of Bierau et al.¹⁴ could be due to the fragmentation peak instead of the condensation peak. Based on the report of Buchenau et al.,¹⁹ the intensity of the fragmentation peak could dominate the overall droplet beam under certain conditions, although in all of our experiments, the maximum contribution of the fragmentation peak is less than 50% of the overall intensity on the Daly detector. During the measurement, the exceedingly large droplets from the fragmentation peak could have masked the existence of the condensation peak without sufficient time resolution.

The size distribution of GFP doped droplets agrees quite well with the pre-doping size of neutral droplets in the temperature range above 7 K. According to Gomez et al.,³⁰ at a stagnation temperature of 7 K and pressure of 20 atm, the average size of a continuous neutral droplet beam should be $\sim 10^7$ atoms/droplet. In our setup, the GFP ions coming out of the ion trap from the ESI source are slowed down by the biased collector/stop electrode, and hence the kinetic energy of the incident ions should be close to zero in the doping region. Considering that each GFP ion carrying +34 charges from an ion trap biased at 2 V requires 10^5 helium atoms to remove its kinetic energy upon colliding with a droplet, and another 10^5 atoms to remove its thermal energy, it is reasonable that the resulting final droplet contains 3.8×10^6 atoms/droplet.

However, at temperatures lower than 7 K, the sizes of our condensation peak are several orders of magnitude smaller than those reported for neutral droplets from continuous sources: at 5 K, the average size reaches $\sim 10^{10}$ atoms/droplet.^{29–31} On the other hand, within our limited power supply for the retardation electrode, we could not resolve the size of the fragmentation peak, although a lower limit of 10^8 atoms/droplet can be deduced. In this sense, the sizes of the large neutral droplets are probably on par with those from our fragmentation peak. We suspect once again that this discrepancy is due to the masking effect of the fragmentation peak in the measurement of neutral droplets. Neither Gomez et al.³⁰ nor Grisenti and Toennies²⁹ were able to resolve the condensation peak from the fragmentation peak, hence it is possible that the reported size is predominantly determined by the fragmentation peak.

From 11 K to 5 K, the expansion process changes from subcritical to supercritical, but the results of Fig. 4 show no abrupt change in velocity, and the size of the condensation peak also remains on the same order of magnitude across the temperature range. The main change is in the magnitude of the fragmentation peak, from barely detectable at 11 K to about equal intensity as that of the condensation peak at 5 K. We therefore believe that as long as the

source is above 5 K, which incidentally is close to the critical temperature of helium,³² there is no major discontinuity in the formation mechanism of superfluid helium droplets. The condensation and fragmentation processes coexist, but the relative intensities of the two vary.

If we assume that a GFP ion is located inside a droplet, a discussion on the geometric dimensions of the ion and a helium droplet, and the feasibility of electron diffraction of GFP doped droplets, is helpful. A pure droplet of size 3.8×10^6 atoms/droplet has a radius of 35 nm.³ Compared with the dimension of a folded GFP molecule,²¹ 2.5 nm in diameter and 4.0 nm in length, the droplet is sufficiently large to completely envelop the GFP ion. If a high-energy electron beam were to penetrate through the diameter of the droplet, it would encounter ~200 helium atoms assuming an interatomic distance of 3 Å for helium,³ and 20 – 40 carbon atoms. The resulting helium to carbon ratio is less than 10:1. The diffraction cross section of carbon and helium for electrons at 40 keV is 40:3,³³ and based on our previous experience, the current ratio is acceptable for effective background removal.³⁴ We therefore conclude that the smaller GFP doped droplets from gas condensation in Fig. 2 are good enough for direct diffraction, while those from liquid fragmentation are probably too large. On the other hand, ion doped droplets do have the advantage of complete separation from undoped droplets using electrostatic steering. With complete removal of undoped droplets in the diffraction region, an even higher tolerance of the helium background is possible.

The separation between the condensation and fragmentation peaks offers an easy and clean size selection method for experiments relying on different sized droplets. Size selection of neutral droplets has been achieved via changes in the expansion conditions^{1–6} or velocity slip in pulsed sources.^{7,9,35} In either case, a clean separation of sizes is difficult if possible at all. The time gap in Fig. 2 is on the order of 400 μs, large enough for almost any pulsed experiments to have a clean selection. In this sense, although the doped droplets in the fragmentation peak contain too many helium atoms, they pose no direct threat to the diffraction experiment because of their slower velocity and hence complete separation from the condensation peak.

Absolute pickup efficiency and number of GFP doped droplets

The difficulty in calibrating the gain of the Daly detector makes the report on the absolute number of ion doped droplets qualitative in nature. Based on the conversion factor obtained from Cs⁺ between the voltage signal on the channeltron of the dynode and the number of droplets on the copper target,¹⁸ an estimate of 700 is obtained for the GFP doped droplets. The absolute doping efficiency as expressed by the ratio of ion doped droplets over the total number of ions from the ESI source is on the order of 10^{-4} , similar to our previous report on cesium ions, reserpine, and substance P.^{17,18} These numbers can be improved by better controlling the ion trajectories in the doping region, and by improving the flux of droplets of the desired sizes. Although intuitively an ion trap should be helpful in recycling the bare ions in the doping region, as adopted by von Helden's group,^{14–16} there seem to be limited improvements in the final number of doped droplets with standard quadrupole or hexapole traps, at least when compared with our results. This is because trapped ions typically possess a certain amount of kinetic energy, and even a moderate amount of energy translates into a

velocity on the order of kilometers/second for a bare ion. To slow down and trap the ion in a droplet, a large number of collisions between the ion and the helium atoms inside a droplet is required.^{17,18} Moreover, the collisional cooling gas necessary in most ion traps can obliterate the droplet beam, hence it has to be pumped out prior to doping. For these reasons, electrostatic cone traps containing no cooling gases and with regions of low kinetic energies are better suited.³⁶

An encouraging observation of the experiment is that the total number of ion doped droplets is not significantly affected by the concentration of acid in the solution. A lower concentration of acid results in a lower average charge of the resulting ions, and since the number of ions in the ion trap prior to doping is limited by the space charge, a lower acid concentration in the spray solution can actually translate into more ions for doping. More importantly, a lower charge state is preferable for maintaining the native folded conformation of the protein by lowering the Coulombic repulsion among surface charges.³⁷ The only experimental difficulty in pursuing the low charge state with a minimal acid concentration in our setup is clogging of the ESI capillary. Innovations in the ESI source for producing low charge more stable ions and for increasing ion fluxes are thus highly desirable.

Conclusion

In this work, we have demonstrated the capability of doping macromolecules into superfluid helium droplets. Given the large heat capacity and kinetic energy of multiply charged GFP ions, a low source temperature below 10 K is required. At these low temperatures, the ion doped droplets exhibit two different velocities with different sizes: the faster group is smaller, corresponding to droplets formed via condensation of gaseous helium, and the slower group is larger, corresponding to droplets formed via fragmentation of liquid helium. These behaviors are in agreement with previous understandings of the droplet formation mechanism. The total number of doped droplets is several hundreds, and the doping efficiency is $\sim 10^{-4}$. From the estimate of the droplet sizes and diffraction cross sections of helium and carbon, electron diffraction from the smaller sized condensation peak should be practical. Improvements in the ESI source should hold the promise of both increasing the ion counts and decreasing the charge states of protein samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by the National Institute of General Medical Sciences (WK, 1R01GM101392-01A1 and RAM, GM114653A) from the National Institutes of Health.

References

1. Toennies JP, Vilesov AF. Matrix Techniques: Superfluid Helium Droplets: A Uniquely Cold Nanomatrix for Molecules and Molecular Complexes. *Angew Chem, Int Ed.* 2004; 43:2622–2648.

2. Stienkemeier F, Lehmann KK. Spectroscopy and Dynamics in Helium Nanodroplets. *J Phys B: At, Mol Opt Phys*. 2006; 39:R127–R166.
3. Slenczka, A., Toennies, JP. Low Temperatures and Cold Molecules. Smith, IWM., editor. World Scientific Press; Singapore: 2008. p. 345-392.
4. Callegari, C., Jäger, W., Stienkemeier, F. Handbook of Nanophysics. Sattler, KD., editor. CRC Press; 2011. p. 4.1
5. Yang S, Ellis AM. Helium Droplets: A Chemistry Perspective. *Chem Soc Rev*. 2013; 42:472–484. [PubMed: 23093018]
6. Toennies JP. Helium Clusters and Droplets: Microscopic Superfluidity and Other Quantum Effects. *Mol Phys*. 2013; 111:1879–1891.
7. Zhang J, He Y, Kong W. Communication: Electron Diffraction of Ferrocene in Superfluid Helium Droplets. *J Chem Phys*. 2016; 144:221101. [PubMed: 27305988]
8. He Y, Zhang J, Kong W. Electron Diffraction of Cbr₄ in Superfluid Helium Droplets: A Step Towards Single Molecule Diffraction. *J Chem Phys*. 2016; 145:034307. [PubMed: 27448887]
9. He Y, Zhang J, Lei L, Kong W. Self-Assembly of Iodine in Superfluid Helium Droplets: Halogen Bonds and Nanocrystals. *Angew Chem, Int Ed*. 2017; 56:3541–3545.
10. Kong W, Pei L, Zhang J. Linear Dichroism Spectroscopy of Gas Phase Biological Molecules Embedded in Superfluid Helium Droplets. *Int Rev Phys Chem*. 2009; 28:33–52.
11. Spence JCH, Doak RB. Single Molecule Diffraction. *Phys Rev Lett*. 2004; 92:198102. [PubMed: 15169448]
12. Leney AC, Heck AJR. Native Mass Spectrometry: What Is in the Name? *J Am Soc Mass Spectrom*. 2017; 28:5–13. [PubMed: 27909974]
13. Marty MT, Hoi KK, Robinson CV. Interfacing Membrane Mimetics with Mass Spectrometry. *Acc Chem Res*. 2016; 49:2459–2467. [PubMed: 27736086]
14. Bierau F, Kupser P, Meijer G, von Helden G. Catching Proteins in Liquid Helium Droplets. *Phys Rev Lett*. 2010; 105:133402. [PubMed: 21230773]
15. Filsinger F, Ahn DS, Meijer G, von Helden G. Photoexcitation of Mass/Charge Selected Hemin⁺, Caught in Helium Nanodroplets. *Phys Chem Chem Phys*. 2012; 14:13370–13377. [PubMed: 22935688]
16. Gonzalez Florez AI, Ahn D-S, Gewinner S, Schoellkopf W, von Helden G. Ir Spectroscopy of Protonated Leu-Enkephalin and Its 18-Crown-6 Complex Embedded in Helium Droplets. *Phys Chem Chem Phys*. 2015; 17:21902–21911. [PubMed: 26234567]
17. Zhang J, Chen L, Freund WM, Kong W. Effective Doping of Low Energy Ions into Superfluid Helium Droplets. *J Chem Phys*. 2015; 143:074201. [PubMed: 26298127]
18. Chen L, Zhang J, Freund WM, Kong W. Effect of Kinetic Energy on the Doping Efficiency of Cesium Cations into Superfluid Helium Droplets. *J Chem Phys*. 2015; 143:044310. [PubMed: 26233132]
19. Buchenau H, Knuth EL, Northby J, Toennies JP, Winkler C. Mass Spectra and Time-of-Flight Distributions of Helium Cluster Beams. *J Chem Phys*. 1990; 92:6875–6889.
20. Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. Engineering and Characterization of a Superfolder Green Fluorescent Protein. *Nat Biotechnol*. 2006; 24:79–88. [PubMed: 16369541]
21. Tsien RY. The Green Fluorescent Protein. *Annu Rev Biochem*. 1998; 67:509–544. [PubMed: 9759496]
22. Miyake-Stoner SJ, Miller AM, Hammill JT, Peeler JC, Hess KR, Mehl RA, Brewer SH. Probing Protein Folding Using Site-Specifically Encoded Unnatural Amino Acids as FRET Donors with Tryptophan. *Biochemistry*. 2009; 48:5953–5962. [PubMed: 19492814]
23. Peeler JC, Mehl RA. Site-Specific Incorporation of Unnatural Amino Acids as Probes for Protein Conformational Changes. *Methods Mol Biol*. 2012; 794:125–134. [PubMed: 21956560]
24. Cooley RB, Karplus PA, Mehl RA. Gleaning Unexpected Fruits from Hard-Won Synthetases: Probing Principles of Permissivity in Non-Canonical Amino Acid-Trna Synthetases. *ChemBioChem*. 2014; 15:1810–1819. [PubMed: 25044993]

25. He Y, Zhang J, Kong W. Electron Impact Ionization and Multiphoton Ionization of Doped Superfluid Helium Droplets: A Comparison. *J Chem Phys.* 2016; 144:084302. [PubMed: 26931697]
26. Pentlehner D, Riechers R, Dick B, Slenczka A, Even U, Lavie N, Brown R, Luria K. Rapidly Pulsed Helium Droplet Source. *Rev Sci Instrum.* 2009; 80:043302. [PubMed: 19405651]
27. He Y, Zhang J, Li Y, Freund WM, Kong W. Facile Time-of-Flight Methods for Characterizing Pulsed Superfluid Helium Droplet Beams. *Rev Sci Instrum.* 2015; 86:084102. [PubMed: 26329210]
28. Christen W, Rademann K, Even U. Supersonic Beams at High Particle Densities: Model Description Beyond the Ideal Gas Approximation. *J Phys Chem A.* 2010; 114:11189–11201. [PubMed: 20961156]
29. Grisenti RE, Toennies JP. Cryogenic Microjet Source for Orthotropic Beams of Ultralarge Superfluid Helium Droplets. *Phys Rev Lett.* 2003; 90:234501. [PubMed: 12857262]
30. Gomez LF, Loginov E, Sliter R, Vilesov AF. Sizes of Large He Droplets. *J Chem Phys.* 2011; 135:154201. [PubMed: 22029306]
31. Knuth EL, Henne U. Average Size and Size Distribution of Large Droplets Produced in a Free-Jet Expansion of a Liquid. *J Chem Phys.* 1999; 110:2664–2668.
32. Brooks JS, Donnelly RJ. The Calculated Thermodynamic Properties of Superfluid Helium-4. *Journal of Physical and Chemical Reference Data.* 1977; 6:51–104.
33. Jablonski, A., Salvat, F., Powell, CJ. Nist Electron Elastic-Scattering Cross-Section, Database, Version 3.2, Srd 64. National Institute of Standards and Technology; Gaithersburg, MD: 2010.
34. Zhang J, He Y, Freund WM, Kong W. Electron Diffraction of Superfluid Helium Droplets. *J Phys Chem Lett.* 2014; 5:1801–1805. [PubMed: 24920997]
35. Yang S, Ellis AM. Selecting the Size of Helium Nanodroplets Using Time-Resolved Probing of a Pulsed Helium Droplet Beam. *Rev Sci Instrum.* 2008; 79:016106. [PubMed: 18248078]
36. Schmidt HT, Cederquist H, Jensen J, Fardi A. Conetrap: A Compact Electrostatic Ion Trap. *Nucl Instrum Methods Phys Res, Sect B.* 2001; 173:523–527.
37. Natalello A, Santambrogio C, Grandori R. Are Charge-State Distributions a Reliable Tool Describing Molecular Ensembles of Intrinsically Disordered Proteins by Native Ms? *J Am Soc Mass Spectrom.* 2017; 28:21–28. [PubMed: 27730522]

Figure 1.

Experiment Setup. Isolated protein ions are produced from the ESI source after passing through a quadrupole mass spectrometer and an ion trap. In the doping region, protein ions are slowed down to traverse the pulsed droplet beam. The doped droplets are subjected to a retardation field for size analysis. A Daly type detector is used for detection of all doped droplets. The copper target located directly downstream from the droplet beam is only used for calibration: to record the total voltage rise due to the doped droplets.

Figure 2.

Arrival times of GFP doped droplets detected on the Daly detector. The stagnation pressure of the pulsed valve was 26 atm. The peaks are called the condensation peak, the rebound peak, and the fragmentation peak based on their time of arrival from earliest to latest.

Figure 3.

Measurements of size distribution of GFP doped droplets (the condensation peak only) at source temperatures of 5, 7, 9, and 11 K and a stagnation pressure of 45 atm. Discrete points are experimental data while continuous lines are single exponential fitting results. The average size and relative intensities are listed in Table 1. The cross-squares are results from the fragmentation peak at a source temperature of 7 K. (a) Ion counts as a function of the retardation voltage. The relative intensities at different temperatures are scaled relative to the maximum at 9 K. (b) Size distribution obtained from (a) by converting the voltage to cutoff size and then to the size distribution of the droplet beam. The maximum at each temperature is normalized to unity. All profiles remain constant in the size range from 0 to 10^5 , so the figure only shows the larger size region to emphasize the size difference at different source temperatures. The uncertainty is 10% for the experimental data.

Figure 4. Velocities of the condensation peak and the fragmentation peak as functions of the source temperature from GFP doped droplets obtained from Fig. 2. Theoretical values were calculated using Eq. 2 in the text. The uncertainty in the experimental values is 10%.

Table 1

Fitting parameters of GFP doped droplets (condensation peak only).

$y=y_0+ Ae^{-\frac{x}{a}}$	5K	7K	9K	11K
Average size a (He atoms, 10^6)	5.7 ± 0.4	3.8 ± 0.3	3.1 ± 0.1	3.3 ± 0.3
Relative total signal	0.83	1.00	0.70	0.44
y_0/A	0.07	0.07	0.04	0.03
Coefficient of determination	0.99	0.98	1.00	0.98