Reducing effects of particle adsorption to the air–water interface in cryo-EM

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Most protein particles prepared in vitreous ice for single-particle cryo-electron microscopy (cryo-EM) are adsorbed to air–water or substrate–water interfaces, which can cause the particles to adopt preferred orientations. By using a rapid plunge-freezing robot and nanowire grids, we were able to reduce some of the deleterious effects of the air–water interface by decreasing the dwell time of particles in thin liquid films. We demonstrated this by using single-particle cryo-EM and cryo-electron tomography (cryo-ET) to examine hemagglutinin, insulin receptor complex, and apoferritin.

Single-particle cryo-EM allows the structural study of purified proteins in solution at near-atomic resolution. To preserve proteins of interest in their hydrated state, one spreads the sample out in a thin layer of buffer solution supported on a cryo-EM grid and rapidly plunges the grid into a cryogen to convert the liquid layer into vitreous ice. Alignment, classification, and reconstruction of a sufficient number of electron microscopy images of randomly oriented protein particles leads to 3D density maps. Advances in electron microscope hardware, cameras, and image-processing methods have made cryo-EM a strong method for reconstructing a wide range of protein complexes to near-atomic resolution in near-native conditions in multiple functional states.

Tomographic studies of single-particle samples have shown that the vast majority of proteins prepared over holey substrates by standard vitrification methods are adsorbed to air–water interfaces. This has the potential to cause the particles to adopt preferred orientations, as well as to damage or degrade the protein structure. There are various ways to avoid protein contact with the air–water interface, including using surfactants as a barrier, sequestering the particles to a support film, and preventing some of the surface effects by reducing the length of time that the sample dwells in the thin liquid film before vitrification. It is this last approach that we describe here; our results suggest that if the dwell time is reduced sufficiently, the particles in solution may either not have time to diffuse completely to air–water or substrate–water interfaces or not equilibrate fully after arriving at the interface, depending on their affinity for the interfaces.

With most cryo-EM vitrification devices (e.g., FEI Vitrobot, Gatan CP3, Leica EM GP, manual plungers), the time that elapses between the wicking of a sample to a thin film and placement of the grid in the cryogen is typically on the order of 1 s or more. With a thin film ~100 nm thick, various estimates indicate that the protein particles will collide with an air–water interface about 100–1,000 times during this time interval, which provides ample opportunity for adsorption and preferential orientation. There are at least three devices currently under development that allow for much more rapid plunge-freezing: a microfluidic spray-plunging machine developed by the Frank group, a surface-acoustic-wave-based microfluidic dispenser from the de Marco group, and Spotiton, a robotic device that dispenses picoliter volumes of sample onto a self-blotting nanowire grid as it quickly moves past en route to vitrification. Here we used Spotiton to achieve rapid plunge times; however, this approach is completely generalizable to other devices. We call the time interval between sample application to the grid and vitrification the spot-to-plunge time. Spot-to-plunge times obtained previously with the Spotiton robot were on the order of 500 ms or more, but we recently modified the device to achieve spot-to-plunge times on the order of 100 ms.

Here we demonstrate, using three different specimens (hemagglutinin, insulin receptor bound to insulin, and apoferritin), that when the spot-to-plunge time and thus the dwell time of the sample in the thin liquid layer are decreased, the number of orientations of particles adsorbed to air–water interfaces may be increased, and the density of nonadsorbed particles in grid holes also may be increased. We used Spotiton to vitrify samples with spot-to-plunge times of 100–200 ms and compared the results, using cryo-EM and cryo-ET, to those for control samples with slower spot-to-plunge times of 400 ms to ~1 s.

With longer spot-to-plunge times (500 ms to ~1 s), hemagglutinin exhibited pronounced preferred orientation, presenting very few side views of the particles in 2D class averages (Fig. 1a), whereas 2D class averages of insulin-bound insulin receptor showed a limited set of particle orientations (Fig. 1b). For both samples, the vast majority of particles were closely associated with air–water interfaces (Supplementary Videos 1 and 2). As a consequence of the preferred orientation, coherent initial models of hemagglutinin and insulin receptor could not be generated and isotropic 3D reconstructions could not be obtained unless micrographs were acquired with the grid tilted relative to the electron beam, which imposed collection, processing, and resolution limitations. Apoferritin with 0.5 mM TCEP (15 mg/ml = 20,530 particles/µm³ in solution), when plunged with a longer spot-to-plunge time of 500 ms, primarily adsorbed to air–water interfaces, although its high symmetry and the prevalence of local tilt in exposure areas effectively negated any issues of preferred orientation (Fig. 2a, Supplementary Video 3). The volume of ice occupied by the nonadsorbed apoferritin particles contained a particle density of about 1,668 particles/µm³ (Supplementary Fig. 1).

A shorter spot-to-plunge time of 100 ms for hemagglutinin resulted in greatly reduced adoption of preferred orientations (Fig. 1a).

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and a 3D reconstructed map at 3.8 Å (Supplementary Fig. 2) that was more isotropic and better resolved compared with the 4.2-Å map produced via grid tilting. Similarly, for the insulin-bound insulin receptor with a spot-to-plunge time of 200 ms, the reduced appearance of the preferred orientation provided additional critical views of the complex (Fig. 1b), thus producing a 4.9-Å 3D reconstruction (Supplementary Fig. 3) that was of higher quality and more isotropic than that derived from images of tilted grids.

Although hemagglutinin and insulin receptor both took on substantially less preferred orientations with shorter spot-to-plunge times, the majority of particles still remained adsorbed to the air–water interfaces (Supplementary Videos 4 and 5). However, in the case of apoferritin with TCEP, a spot-to-plunge time of 170 ms dramatically increased the density of nonadsorbed particles compared with that observed with a time of 500 ms (Fig. 2b, Supplementary Video 6). The density of particles not adsorbed to the air–water interfaces increased by a factor of ~20x to about 31,725 particles/µm³ (Supplementary Fig. 4). When apoferritin at a lower concentration was prepared without TCEP (6 mg/ml = 8,212 particles/µm³ in solution), the density of nonadsorbed particles increased from about 3,043 particles/µm³ with a spot-to-plunge time of 500 ms to about 17,927 particles/µm³ with a spot-to-plunge time of 100 ms (Supplementary Figs. 5 and 6, Supplementary Videos 7 and 8). We note that far fewer particles adsorbed to the air–water interface in the apoferritin sample with TCEP added to the buffer, but it is likely that additional factors contributed to the observed differences. These factors include effects of evaporation, estimated at 300 Å/s (for ~85% relative humidity, ~70 °F). A full understanding of these and other effects clearly requires much further study under well-defined and controlled conditions.

These three example specimens showed that deleterious effects of particle adsorption to air–water interfaces can be considerably reduced by minimization of the time between sample application and plunge-freezing, which increases the density of nonadsorbed particles in holes and decreases the number of opportunities for particles to equilibrate at air–water interfaces. We anticipate that the use of even shorter spot-to-plunge times, in the range of a few tens of milliseconds, would reduce the interface effects further. Our observations in this study do not directly address whether faster plunging would result in fewer particles being adsorbed to the air–water interface, but they do clearly indicate that the overall effect is reduced adoption of preferred orientations. In the case of Spotiton, the use of faster spot-to-plunge times could make it more challenging to control ice thickness and might require higher-mesh grids (with increased nanowire density) or more accurate control of the relative humidity. However, although thinner ice is usually the ideal outcome, we note that near-atomic-resolution structures are attainable in ice thicker than 100 nm (ref. 19). Finally, if the density...
of nonadsorbed particles can be significantly increased, per-particle contrast transfer function estimation\(^{20-22}\) on the resulting images will provide the possibility for in silico identification of nonadsorbed versus adsorbed particles in single-particle cryo-EM. As a result, it might be possible to derive single-particle cryo-EM structures on the basis of only nonadsorbed particles, which would allow for more explicit studies of the effects of adsorption on structure and preferred orientation.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41592-018-0139-3.

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Author contributions
A.J.N. collected and aligned tilt series and analyzed cryo-ET data. H.W. and V.P.D. performed the Spotion experiments and the cryo-EM experiments. H.W. made nanowire grids and collected tilt series. H.W., V.P.D., and Y.Z.T. analyzed single-particle cryo-EM data. A.J.N., H.W., V.P.D., Z.Z., C.S.P., and B.C. conceived and designed the experiments. A.J.N. and B.C. wrote the manuscript.

Competing interests
B.C. and C.S.P. have a commercial relationship with TTP Labtech, a company that will produce a commercially available Spotion instrument.

Additional information
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Apoferritin. For the experiments in Fig. 2, Supplementary Figs. 1 and 4, and Supplementary Videos 3 and 6, we prepared apoferritin from equine spleen (Sigma-Aldrich) by diluting the stock sample to 15 mg/ml with 0.5 mM TCEP.

For the experiments shown in Supplementary Figs. 5 and 6 and Supplementary Videos 7 and 8, we prepared apoferritin from equine spleen (Sigma-Aldrich) as follows. 100 µl of sample at 25 mg/ml was diluted with 1 ml QA (20 mM HEPES, pH 7.5, 0.1 mM EDTA, pH 8.0, 1 mM DTT), 1 ml was loaded into a Q column and eluted with QB (QA + 1.5 M NaCl), and the main peak was pooled and concentrated to 0.5 ml. This 0.5 ml was then loaded onto a 100000S200 column (GE) in QE (150 mM NaCl, 20 mM HEPES, pH 7.5), eluted at approximately 10.5 ml, and concentrated to working conditions.

Grid preparation and vitrification. Nanowire grids were prepared as described by Raininko et al.12 and Wei et al.13.

The samples were vitrified with the semi-automated Spotiton V1.0 robot, a novel device for preparing cryo-EM samples by means of piezo-dispersing small (50-µl) drops of sample across a ‘self-blotting’ nanowire grid as it moves past before being plunged into liquid ethane. We used nanowire grids, manufactured in-house, backed by lacey carbon film supports for all experiments. Nanowires were dispensed on a grid dropping vertically past the dispense head in 50-pl drops for a total of ~5 nL of sample applied as a stripe across the grid, which was then plunged into liquid ethane. The time between application of sample to the grid and plunging of the grid into liquid ethane (spot-to-plunge time) ranged from 100 to 800 ms (ref. 14). Spotiton was operated at ~85% relative humidity and ambient temperature (~70°F). Under these conditions, evaporation is estimated as 300 Å/s. The time of flight of the 50-µl drops from the nozzle to the grid is estimated as 0.5 ms, and only the first drop has time to form a skin ‘as it waits in the nozzle. Thus, this phase is probably unimportant for the formation of the air–water interface. After the first contact of drops to grid, the drops have a fairly large volume relative to the surface area and spread out under their own momentum. Contact with the nanowires results in very rapid (~20 ms) formation of a thin layer of a water–air interface. Therefore, we estimate that our counting accuracy for this one grid; therefore, although we estimate that our counting accuracy for this one

Single-particle cryo-EM data collection. Hemagglutinin. Single-particle micrographs were collected on a Titan Krios (Thermo Fisher Scientific) equipped with an energy filter and a K2 counting camera (Gatan, Inc.). Sample was dispensed on a grid dropping vertically past the dispense head in 50-pl drops for a spot-to-plunge time ranging from 100 to 800 ms (ref. 14). Spotiton was operated at ~85% relative humidity and ambient temperature (~70°F).

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Single-particle cryo-EM data processing. Hemagglutinin. Single-particle micrographs were collected on a Titan Krios (Thermo Fisher Scientific) equipped with an energy filter, C2 corrector, and K2 counting camera (Gatan, Inc.); the microscope was operated at 300 kV at a nominal magnification of 130,000x, with a calibrated pixel size of 1.081 Å. Exposure was set to 10 s (40 frames per image), for a total dose of 73.5 e/Å². The samples were archived with a defocus range of 1–2.5 µm.

Cryo-ET tilt-series data processing. We aligned tilt series using Appion-Protoom36,37 by dose-compensating the images with respect to the total accumulated dose38 of the tilt series, coarse-aligning tilt images, manually fixing coarse alignment if necessary, refining tilt-series alignment over several dozen iterations, and reconstructing with Tomo3D SIRT39,40. Tilt series were not CTF-corrected.

Cryo-ET tilt-series data collection. Tilt series were collected on a Titan Krios (Thermo Fisher Scientific) equipped with an energy filter and a K2 counting camera (Gatan, Inc.) and on a Tecnai F20 (Thermo Fisher Scientific) with a DE-20 camera (Direct Electron). Most tilt series were collected nominally from ~45° to 35° with a small fixed or Saxton scheme41 increments in either Leginon23 or SerialEM42 with a nominal defocus near 5 µm, per-tilt image doses between 2 and 3 e/Å², and pixel sizes of 2.16 Å (K2) and 2.34 Å (DE-20). K2 tilt images were whole-frame aligned using MotionCor2.

Tomogram particle-picking. Particles in the apoferritin tomograms (Supplementary Videos 3 and 6–8) were picked manually using Dynamo43. Particle picks were categorized by location: (1) particles over the carbon in the 0° projection direction or adsorbed to the carbon, (2) particles adsorbed to the air–water interfaces in the grid holes, and (3) nonadsorbed particles in the grid holes. The picks on the volume provided an approximation to within 1%, whereas volume measurements are accurate to within 5% owing to ice curvature and grid-shape approximations; thus density calculations are likely to be accurate to within 5%. Density calculations were performed on only one tomogram (one location on the grid) per apoferritin grid; therefore, although we estimate that our counting accuracy for this one tomogram is 5%, this is clearly not applicable across the entire grid.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Single-particle half-maps, full sharpened maps, and masks for insulin-bound insulin receptor (200- or 600-ms spot-to-plunge time) and hemagglutinin (100-ms spot-to-plunge time) have been deposited in the Electron Microscopy Data Bank (EMDB) with accession codes EMD-7788, EMD-7791, and EMD-7792, respectively. The full single-particle collection of hemagglutinin with 100-ms and 500-ms spot-to-plunge times has been deposited in the Electron Microscopy Pilot Image Archive (EMPIAR) with accession codes EMPIAR-10175 and EMPIAR-10197, respectively. Single-particle cryo-ET tomograms have been deposited in the EMDB with accession codes EMD-7623, EMD-7624, EMD-7625, EMD-7710, EMD-7627, EMD-7628, EMD-7629, and EMD-7630. Single-particle cryo-ET tilt series, cryo-ET tilt-series alignment runs with Appion-Protoom, cryo-ET tomograms, and apoferritin particle picks have been deposited in the EMPIAR with accession codes EMPIAR-10169, EMPIAR-10170, EMPIAR-10171, EMPIAR-10141, EMPIAR-10172, EMPIAR-10129, EMPIAR-10173, and EMPIAR-10174.

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Software and code

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Data collection

Legionov v3.3 beta was used to collect single particle micrographs and tilt-series. SerialEM was used to collect tilt-series.

Data analysis

CryoSPARC v0.6.5 was used for single particle alignment and classification. Appion-Protomo, using Appion v3.3 beta and Protomo v2.4.1, was used to align tilt-series.

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Single particle half maps, full sharpened maps, and masks for insulin-bound insulin receptor (200 ms spot-to-plunge time), insulin-bound insulin receptor (800 ms), and hemagglutinin (100 ms) have been deposited to the Electron Microscopy Data Bank (EMDB) with accession codes EMD-7788, EMD-7791, and EMD-7792.
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Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Three samples (apoferritin, hemagglutinin, and insulin-bound insulin receptor) were selected from in-house projects due to their observed behavior when plunging faster, as described in the manuscript. No sample size calculation was performed. Three samples were chosen in order to show applicability to: 1) a pathologically preferentially-oriented particle (hemagglutinin), 2) a novel particle (insulin-bound insulin receptor), and 3) a canonically-shaped particle (apoferritin). |
|---|---|
| Data exclusions | No data was excluded. |
| Replication | Both insulin receptor and hemagglutinin were used to show reproducibility with regards to reducing preferred orientation issues. Both apoferritin and apoferritin with 0.5mM TCEP were used to show reproducibility with regards to increasing the density of non-adsorbed particles. All attempts to replicate these two results with these samples have thus far been successful. |
| Randomization | This is not relevant in this study because protein structural data could not be randomized. |
| Blinding | Blinding is not relevant to this study because the relevant tests - preferred orientation and a change in non-adsorbed particle density - were performed in a manner that does not introduce bias. Preferred orientation was analyzed with exclusively software-based 2D classification and 3D reconstruction. Non-adsorbed particle density was analyzed by explicitly counting non-adsorbed particles and measuring the volume they occupy. The raw data together with the particle picks are provided together with the manuscript. |

Materials & experimental systems

Policy information about availability of materials

n/a Involved in the study

- Unique materials
- Antibodies
- Eukaryotic cell lines
- Research animals
- Human research participants

Method-specific reporting

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