Amorphous nickel titanium alloy film: a new choice for cryo electron microscopy sample preparation

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ABSTRACT

Cryo-electron microscopy (cryoEM) has become one of the most important approach for structural biology. However, barriers are still there for an increase successful rate, a better resolution and improved efficiency from sample preparation, data collection to image processing. CryoEM sample preparation is one of the bottlenecks with many efforts made recently, including the optimization of supporting substrate (e.g. ultra-thin carbon, graphene, pure gold, 2d crystal of streptavidin, and affinity modification), which was aimed to solve air-water interface problem, or reduce beam induced motion (BIM), or change particle distribution in the grid hole. Here, we report another effort of developing a new supporting substrate, the amorphous nickel-titanium alloy (ANTA) film, for cryoEM sample preparation. Our investigations showed advantages of ANTA film in comparison with conventional carbon film, including superior electron conductivity and trace non-specific interaction with protein. These advantages yield less BIM and significantly improved particle distribution during cryoEM experiment of human apo-ferritin, thus resulting an improved reconstruction resolution from a reduced number of micrographs and particles. Unlike the pure gold film, the usage of the ANTA film is just same with the carbon film, compatible to conventional automatic cryoEM data collection procedure.

KEYWORDS

Amorphous nickel titanium alloy; beam induced motion; cryoEM sample preparation; particle distribution; supporting film;

1 INTRODUCTION

2 With breakthrough and developments in the past ten years, cryo-electron
3 microscopy (cryoEM), especially cryoEM single particle analysis (SPA), has
4 become a powerful technique to study the high resolution three dimensional
5 structure of biological macromolecules in solution (Cheng et al., 2017). However,
6 there are still many bottlenecks to overcome for the future wide application of this
7 technique. One of the most important bottlenecks is how to routinely prepare
8 suitable samples for high resolution data collection and the following structure
9 determination (Glaeser, 2016).

10 During cryoEM sample preparation, we had many issues to challenge,
11 including protein denature/degradation and preferred orientation induced by the
12 air-water interface (Glaeser et al., 2016), non-uniform/ill distribution of protein
13 particles in the hole (Drulyte et al., 2018), un-expected few number of particles in
14 the hole (Snijder et al., 2017), not well controlled ice thickness and etc. These
15 challenges increase difficulties and efficiencies for the subsequent cryoEM data
16 collection and thus become the barrier of a better resolution. The way of cryoEM
17 sample preparation also affects another important factor, beam induced motion
18 (BIM), which is critical for high resolution cryoEM imaging (Brilot et al., 2012).

19 Many efforts have been made in the past to solve these challenges by
20 improving cryoEM sample preparation. One approach is adjoining a layer of
21 continuous film, e.g. ultra-thin carbon (Grassucci et al., 2007), graphene (Fan et
22 al., 2019; Naydenova et al., 2019; Pantelic et al., 2012; Russo and Passmore,
23 2014a) or graphene derivate (Pantelic et al., 2010), and 2D crystal of streptavidin
24 (Han et al., 2016; Wang et al., 2008) et al., on the top of the holey supporting film.
25 This approach can solve air-water interface issue, alter particle distribution and
26 improve BIM while additional experimental procedures are needed and the
27 additional layer adds another noisy background of the final cryoEM image.
28 Another approach is modifying the surface property of the holey supporting film,
29 e.g. by glow discharging (Isabell et al., 1999), special treatment with surfactants
30 or PEG (Meyerson et al., 2014) or antibody (Yu et al., 2016). This approach can

improve the particle distribution in the hole. To overcome un-expected few number of particles in the hole, applying sample a number of times to saturate the interaction between the sample and the supporting film was successful (Snijder et al., 2017). In addition, besides the conventional carbon film, another attempt to change the supporting substrate to pure gold film was also successful for an extremely reduce BIM (Russo and Passmore, 2014b), while the polycrystalline property of the gold film adds another issue during routine cryoEM data collection. Besides difficulty of TEM alignment, the cryoEM image should be taken in the middle of the hole without the film edge when the pure gold film was used.

In this study, we developed a new type of metal supporting film, amorphous nickel-titanium alloy (ANTA) film, for another good choice of cryoEM sample preparation. The relevant product has been successfully used in real high resolution cryoEM applications (Hua et al., 2020; Wu et al., 2019). Our present studies further showed advantages of ANTA film in comparison to carbon film and pure gold film.

MATERIALS AND METHODS

Preparation of ANTA film and amorphous carbon film

The procedure of preparing supporting film with microarray holes was modified from the published method (Quispe et al., 2007). As shown in **Figure 1A**, the super elastic nickel titanium alloy wire of 0.5 mm diameter (TIANRUI NONFERROUS METAL, Beijing), which is consisted of 40 ~ 60 atomic percent of nickel and 60 ~ 40 atomic percent of titanium, was used to prepare the ANTA film. The wire was heated up to 1500 ~ 2500 °C in a tungsten wire basket and evaporated to a silicon wafer with microarray holes. The evaporation was performed in an oil-free vacuum system (Beiyichuangxin, Beijing) with a high vacuum ($< 5 \times 10^{-4}$ Pa). The ANTA film was formed on the surface of silicon wafer.

The preparation of amorphous C film was made by heating the graphite rod (Ted Pella, Lot#052514-17029) and evaporated at a similar condition to the preparation of ANTA film. The desired thickness of the supporting film was controlled between 18~25 nm.

In order to release the supporting film, the silicon wafer was immersed into the pure water (**Figure 1B**). The air-water interface promotes the supporting film peel off and then float on the water surface. TEM grids were placed under the water surface and then coated with the supporting film by lowering the water level to allow grids attach to the film. Once the coated grids were taken out of water, they were dried and stored in cool and clean environment away from light (**Figure 1C**). The supporting film were examined using FEI Talos F200C transmission electron microscope (ThermoFisher Scientific) at 200kV with 4k*4k Ceta CMOS camera (**Figure 1D**).

Film thickness measurement

Film thickness measurement was performed on a Nanoscope IIIa, MultiMode system SPM (Digital Instruments, Bruker) in a tapping mode and in air. Ultra-sharp NSC11/AIBS silicon cantilevers (MikroMasch, Tallinn, Estonia) with spring constants of ~48 N/m and resonant frequencies of ~330 kHz were used for imaging. Images were collected at a speed of 1 Hz with a resolution of 512x512 pixels. Two types of films were used for thickness measurement, the consecutive film and the microarray-hole one. The consecutive film was prepared by evaporating onto the freshly exposed surface of mica. To create a sharp edge for measurement, single sided Kapton Tape (Ted Pella, Prod No. 16090-6) was attached to the mica to cover the surface partially and removed after evaporation. The microarray-hole film was transferred to mica after peeling off from the silicon wafer. The existence of the holes allows the measurement at any place of the film. After imaging three random regions, flatten and section analysis were carried out using the Nanoscope analysis software to determine the thickness of the film.

Electronic resistivity measurement

The nickel titanium alloy or amorphous carbon was evaporated onto the polished surface of a single-crystal silicon wafer. The thickness of the film was controlled as 30 ± 3 nm and the size of the film was made as 1.8 cm × 1.8 cm. The resistance of the film was measured by four-wire resistance measurement using a physical property measurement system (Quantum Design, San Diego, CA). The

distance between electrodes was kept 1 cm. The temperature variation was coupled with the resistance measurement from 300 K to 77 K and then back to 300 K. The resistivity was calculated according to the formula $\rho = RS/L$, where R is the measured electrical resistance, S is the cross-sectional area and L is the distance between the electrodes.

Cryo sample preparation

TEM grids coated with ANTA film or carbon film were plasma cleaned in Gatan Solarus with the recipe of H_2/O_2 for 1min. 1.3 mg/ml human apo-ferritin (Hfn) in the buffer containing 50 mM Tris-HCl (pH8.0) and 150 mM NaCl was plunge frozen onto TEM grids using Leica EM GP. For the ANTA film, it is recommended to use a less force to touch the filter paper in comparison with carbon film, otherwise the ANTA film might peel off and attach to the filter paper.

Ryanodine receptor 1 (RyR1) was purified as the previous report (Wei et al., 2016) and stored in the buffer containing 20 mM HEPES (pH 7.2), 300 mM NaCl, 0.15 mg/ml amphipol and 2 mM DTT. Vitrification was performed using Vitrobot Mark IV (ThermoFisher Scientific) under 4 °C and 100% humidity. 3 μ l of RyR1 at about 15 mg/ml was applied to ANTA copper 300-mesh grid or R1.2/1.3 Quantifoil 300-mesh copper grid, blotted for 5 s, and then plunge-frozen in liquid ethane.

Measurement of beam induced motion (BIM)

BIM of each ANTA film and carbon film were measured using Hfn sample vitrified and the raw data were captured on FEI Titan Krios transmission electron microscope (ThermoFisher Scientific) at 300 kV with Falcon III camera (ThermoFisher Scientific) in pixel size of 1.42 Å. For each grid, 45 image stacks were taken at the centers of the corresponding holes from 3~4 squares. Squares were selected from the center and four corner areas of the whole grid. Three grids of each group were used for the statistics. All image stacks were taken one minute after stage movement or stage tilt with the dose rate of 24 $e/\text{\AA}^2/\text{s}$, the exposure time of 1.1 s, the defocus range of -2 to -3 μ m and the illumination area of ~ 2 μ m to cover the entire hole. The root of mean square (RMS) of the sample displacement was calculated according to the previous method (Zheng et al.,

1 2017).

2 **CryoEM SPA data collection and processing**

3 The cryoEM SPA datasets of Hfn sample were collected automatically using
4 SerialEM (Mastronarde, 2005) on FEI Titan Krios transmission electron
5 microscope (ThermoFisher Scientific) at 300 kV with Gatan K2 Summit camera.
6 The pixel size was 0.41 Å in the super resolution mode, corresponding to the
7 physical pixel size of 0.82 Å. Each image was exposed for 5.2 sec in a dose rate
8 of 11.5 e/Å²/sec, resulting in a total dose of ~ 60 e/ Å². Each movie stack
9 comprises 32 frames. The defocus range was set from -1 µm to -2.5 µm.

10 All movie stacks were motion-corrected, dose-weighted, distortion-corrected
11 and binned to 0.82 Å by MontionCor2 (Zheng et al., 2017). CTF (Contrast
12 Transfer Function) estimation was performed using CTFFIND4 (Rohou and
13 Grigorieff, 2015). The subsequent data processing including particle picking, 2D
14 and 3D classification, and 3D refinement was performed by using RELION 2.1
15 (Kimanius et al., 2016) and RELION 3.0 (Zivanov et al., 2018).

16 For the dataset using carbon film, 2,177 particles were manually picked from
17 43 micrographs and processed through reference-free 2D classification. Seven
18 2D class-averaged images were chosen as the references for automatic particle
19 picking. In total, more than 90,000 particles were auto-picked from 747
20 micrographs. Then reference-free 2D classification and manual screening were
21 carried out to remove overlapping or degraded particles. 64,667 particles were
22 kept, extracted with a box size of 224 pixels and subjected into 3D
23 auto-refinement, yielding a map with 3.2 Å resolution. After local CTF refinement,
24 beam-tilt correction and particle re-extraction with box size of 288 pixels, the
25 resolution was improved to 2.8 Å. The final map resolution was 2.6 Å after post
26 processing. All reported resolutions were estimated based on the gold-standard
27 FSC (Fourier Shell Correlation) at 0.143. All refinements were started from a 60 Å
28 low-pass filtered initial model (PDB code, 2FHA).

29 For the dataset using ANTA film, the same workflow was applied. Briefly,
30 2,090 particles were initially manually picked from a subset and classified to

create references for auto-picking. In total, more than 110,000 particles were auto-picked from 426 micrographs. After reference-free 2D classification and manual screening, 91,537 particles were selected for 3D refinement. The first round of 3D auto-refinement yielded a map of 3.0 Å resolution. After local CTF refinement and beam-tilt correction, the final resolution of the post processed map was improved to 2.4 Å.

Model building and refinement

The atomic model of Hfm from the crystal structure (PDB code, 2FHA) was first fitted into the final cryoEM map as the initial model using Chimera (Pettersen et al., 2004). Then the real-space refinement was performed using PHENIX (Adams et al., 2010). The refined model was transformed into MRC files, and calculated FSC curves between the model and map by using software EMAN2 (Tang et al., 2007). The map visualization and segmentation were performed by software Chimera.

Henderson-Rosenthal plot

To fairly compare cryoEM SPA results from the support of ANTA and carbon films, for each dataset, 45,211 particles were randomly selected by covering an even range of defocus and orientation distribution. Then partial (1%, 2%, 3%, 4%, 5%, 7%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%) datasets of total particles were selected. Reconstruction was then performed based on the specific subset of selected particles and the map resolution was estimated using the gold standard FSC_{0.143} criteria. Then the Henderson-Rosenthal plots for both datasets were generated as described (Stagg et al., 2014).

Magnetic moment measurement of ANTA film

To measure the magnetic moment of ANTA film, the ANTA film was prepared on a single-crystal silicon wafer with the polished surface and the thickness of the film was controlled as 22 ± 3 nm and the size of the film was made as 1.8 cm × 1.8 cm. Then the magnetic moment was measured by SQUID (Superconducting Quantum Interference Device) using Magnetic Performance Measurement

System (MPMS, Quantum Design Company) at the temperature of 77 K in the environmental magnetic strength of 0.0 T and 3.0 T, respectively. The magnetic moment of the silicon wafer in the same size was measured separately in the same condition as a control.

Protein adhesion test with single molecule force spectroscopy

The carbon and ANTA were separately evaporated onto the clean cover slips (Fisherbrand Microscope cover glass, 12-545-C, 22X40-1, Fisher Scientific). The coating thickness was controlled as 30 nm. Single molecule force spectroscopy (SMFS) was performed using a home-built AFM as described previously (Lee, C. Y., et al., 2013). Biotinylated AFM cantilever (OBL-10, Bruker, Santa Barbara, CA) was ligated with the biotinylated human programmed death-ligand 1 (hPD-L1) by soluble formed streptavidin protein. The hPD-L1 ligated cantilever was provided by Dr. Jizhong Lou's lab (Institute of Biophysics, CAS). Before each experiment, the hPD-L1 ligated cantilever was calibrated in PBS solution, showing a spring constant of around 4 pN/nm. The rupture force between hPD-L1 and the supporting film was measured by force ramp. In one measurement cycle, the AFM cantilever was brought into contact the supporting film for 200 ms, and then was retracted at 200 pN/s. The peak force was identified as the rupture force of the interaction between hPD-L1 and the supporting film.

Hippocampal neuron cell culture on supporting films

The TEM grids coated with ANTA or carbon films were test for their ability of cell culture. The grids were placed in the culture dish with the side of the film up, sterilized under ultraviolet light overnight and coated with the Matrigel matrix (Product #354248, Corning, USA) just before usage.

The primary hippocampal neuron cells were separated from postnatal day 0 to day 1 Sprague-Dawley rats. The protocol of cell culture was modified from the published report (Kang et al., 2008). Hippocampal neurons were separated in HBSS buffer (H2387, Sigma) containing 20% FBS at 4 °C, then digested in the DNase/trypsin solution (0.5 mg/ml DNase, 5 mg/ml trypsin, 25 mM HEPES, 137 mM NaCl, 5 mM KCl, 7 mM Na₂HPO₄, pH 7.2) for 5 min at 37 °C. After the

1 digestion, the single neuron cells were suspended in the DNase solution
2 (0.5 mg/ml DNase, 25 mM HEPES, 137 mM NaCl, 5 mM KCl, 7 mM Na₂HPO₄, pH
3 7.2), and washed with HBSS containing 10% FBS.

4 After that, the primary single neuron cells were plated on the top of grids in the
5 culture dish. The culture dish was kept at 37°C in 5% CO₂ for one week. Culture
6 media (DMEM supplemented with 10% fetal bovine serum, 1% penicillin and
7 streptomycin) was changed every other day by refreshing half of the medium. The
8 optical images of primary hippocampal neuron cells were acquired using an invert
9 fluorescence microscope (Olympus IX81) with the 10X objective and sCMOS
10 camera (Andor Zyla).

11 **RESULTS**

12 **Thickness of ANTA film**

13 The thickness of ANTA film is controlled by the distance between the source
14 and the target as well as the length of the nickel titanium wire that will be depleted
15 after the evaporation process. The exact thickness of film can be measured by
16 atomic force microscope (Nanoscope System, South Korea) (**Figure 2**). Our
17 measurements showed the thickness of ANTA film can be controlled between 18
18 to 25 nm when the distance from the source to the target is set to 9.0 cm and the
19 length of the nickel titanium wire is 1.1 cm.

20 **Low electrical resistivity of ANTA film at liquid nitrogen temperature**

21 Compared to carbon film with the thickness of 30±3nm, the electrical
22 resistivity of which increases significantly from 0.5×10⁻²Ω•m to 5×10⁻²Ω•m with the
23 temperature decreases from 300 K to 77 K, the electrical resistivity of ANTA film is
24 4-order lower and does change significantly with the temperature, from
25 3.4×10⁻⁶Ω•m at 300 K to 3.8×10⁻⁶Ω•m at 77 K (**Figure 3A**). The superior electrical
26 conductivity of ANTA film would yield a reduced beam induced motion (BIM)
27 during cryoEM experiments in comparison with carbon film, which was proved
28 from the dose dependent sample displacement measurements at both 0-degree
29 stage tilt (**Figure 3B**) and 45-degree stage tilt (**Figure 3C**).

1 **Trace magnetic moment of ANTA film**

2 Because of containing the ferromagnetic element of nickel, it would be
3 suspected that ANTA film would generate magnetic field that will affect electrons
4 and thus influence cryoEM imaging significantly. Therefore, we measured the
5 average magnetic moment of ANTA film on a silicon wafer. The experiments were
6 performed in 77 K temperature to mimic the cryogenic condition. At the
7 environmental magnetic strength of 0 T, the measured average magnetic moment
8 in 148 s was -5.17×10^{-6} emu with standard deviation of 9.18×10^{-9} emu while at
9 the environmental magnetic strength of 3 T, which mimics the environment inside
10 objective lens of TEM, the measured average magnetic moment in 261 sec was
11 4.83×10^{-4} emu with standard deviation of 5.95×10^{-7} emu. As the control, the
12 measured average magnetic moment of the silicon wafer in 242 sec at 0 T was
13 1.66×10^{-9} emu with the standard deviation of 7.93×10^{-9} emu, while at 3 T in 305
14 sec was -3.84×10^{-5} emu with the standard deviation of 4.61×10^{-7} emu. Our
15 results showed that ANTA film has a trace magnetic moment, which will not yield
16 obvious effects on cryoEM imaging.

17 **ANTA film is amorphous and suitable for routine TEM alignment**

18 Previous studies have demonstrated the superior property of gold film with
19 much less BIM and better for high resolution cryoEM SPA work. However, the
20 polycrystalline property of gold film makes routine TEM alignment (including
21 coma-free alignment and stigmatism adjustment) on gold film impossible (**Figure**
22 **4**), thus yielding additional difficulties for normal cryoEM SPA data collection.
23 However, as another metal film, ANTA film is amorphous according to its electro
24 diffraction pattern and the power spectrums of ANTA film images show the clear
25 thon rings in response to the change of defocus values, which is just similar to
26 carbon film (**Figure 4**). As a result, not like gold film, the ANTA film can be used in
27 a same way with carbon film and suitable for defocus estimation, stigmatism
28 adjustment and coma-free alignment.

29 **ANTA film yields better cryoEM SPA results in comparison with carbon film**

Based on the reduced BIM of ANTA film in comparison with carbon film, we can predict cryoEM SPA experiments using ANTA film would yield a better result. Here, we selected Hfn as a test sample and collected cryoEM SPA datasets of the same batch of Hfn sample that were vitrified onto ANTA and carbon films, respectively. The final resolution of Hfn reaches 2.4 Å from 426 micrographs for ANTA film while that for carbon film reaches 2.6 Å from 747 micrographs (**Figures 5A, 5B and 5C**). To make a fair comparison, the same number of particles was selected from each dataset and the reconstruction was performed respectively. When the resolution for the carbon film dataset reaches 2.9 Å, the one for the ANTA film from the same number of particles reaches 2.7 Å (**Figure 5D**). The subsequent Henderson-Rosenthal analysis (**Figure 5E**) further confirmed that ANTA film can yield a better result using the same number of particles in comparison with carbon film.

Less none-specific protein absorption of ANTA film

We were interested to observe for ANTA film dataset more particles (~110,000) can be picked from less (426) micrographs in comparison with carbon film dataset where ~90,000 particles were picked from 747 micrographs. When we go back to investigate individual micrographs, we found the particle distribution in ANTA film dataset is more even and less aggregated in comparison with carbon film (**Figures 6A-6D**). We further selected another sample RyR1 to test this phenomenon and found (**Figures 6E and 6F**) there are more particles (~ 100) consistently in the hole for the ANTA film grid while only a few (10 ~ 30) can be picked for the carbon film grid (Quatifoil R1.2/1.3).

We thought this phenomenon might be due to a less none-specific protein absorption of ANTA film in comparison with carbon film. For carbon film, more protein particles are presumably absorbed by carbon film and such absorption would also induce aggregation of the particles. For prove this concept, we performed the single molecule force spectroscopy to measure the interaction force between protein and the supporting film. In the current experiment (**Figure 7**), we selected human programmed death-ligand 1 (hPD-L1) as one testing

example. The results showed that the averaged dissociation force was 16.6 pN between hPD-L1 and the carbon film while the force between hPD-L1 and ANTA film was only 0.94 pN (**Figure 7**).

Good biocompatibility of ANTA film

The above experiments have showed that ANTA film is suitable for cryoEM SPA experiments and better than carbon film. We ought to investigate the possibility of ANTA film for the application of *in situ* cryo-electron tomography and whether it is toxic for cell culturing. We tried to culture rat hippocampal neuron cells, very sensitive to toxic environment, onto the gold grids coated with ANTA and carbon films, respectively. The results showed that the cells can grow and differentiate well on both films (**Figure 8**), demonstrating a similar biocompatibility of ANTA film to carbon film.

DISCUSSIONS

In the present study, we developed a new supporting film for cryoEM experiments, the amorphous nickel-titanium alloy film (ANTA film). As a metal supporting film, the electrical conductivity of ANTA film is much greater than carbon film especially at liquid nitrogen temperature. Therefore, ANTA film exhibits less BIM than carbon film. As a result, in cryoEM SPA application using the support of ANTA film, we could achieve better reconstruction resolution from the same number of particles in comparison with the support of carbon film.

Compared to the pure gold film, another currently widely used metal supporting film, ANTA film is amorphous not polycrystalline, which enables us to perform routine TEM alignment (focus estimation, stigmatism correction and coma-free alignment) directly on the supporting film in a similar way to carbon film, and compatible to the current automatic cryoEM SPA data collection strategy.

However, according to the previous research (Russo and Passmore, 2014), the BIM effect using ANTA film is still a bit higher than the pure gold film, which would be explained by two reasons. First, the electrical conductivity of gold is better than ANTA, about two orders of magnitude lower in the electrical resistivity.

1 Second, ANTA film is coated onto a gold/copper grid and the slightly different
2 thermal expansion coefficient between the supporting film and the grid would
3 induce mechanical instability after vitrification while the pure gold grid does not
4 have such problem.

5 The thermal expansion coefficient of ANTA film is expected to be between
6 nickel ($13.4 \times 10^{-6} \text{ K}^{-1}$) and titanium ($8.6 \times 10^{-6} \text{ K}^{-1}$). The thermal expansion
7 coefficient of block nickel titanium alloy with a similar atomic ratio is around
8 $11 \times 10^{-6} \text{ K}^{-1}$. To reduce cryo-crinkling during vitrification, the selected material of
9 grid should have a similar thermal expansion coefficient with the ANTA film. The
10 grid made of stainless steel ($11.8 \times 10^{-6} \text{ K}^{-1}$) is most suitable for ANTA film coating.
11 The grids made of nickel and titanium are also acceptable. In general, the gold
12 grid with the thermal expansion coefficient of $14.2 \times 10^{-6} \text{ K}^{-1}$ can also be used and
13 it is better than the copper grid with the thermal expansion coefficient of 16.5×10^{-6}
14 K^{-1} . To be noted, the above thermal expansion coefficients are looked up from
15 WebElements (<http://www.webelements.com/>).

16 The high contrast of ANTA supporting film during cryoEM experiments makes
17 identification of the hole area much easier (**Figure 1D**), which is because the
18 atomic numbers of both nickel and titanium are higher than the elements of
19 biological sample. However, it is harder to distinguish the thickness of ice in the
20 hole by comparing the contrast of the hole area with the film area. The similar
21 effect can be also observed when using the pure gold grid.

22 The most interesting finding of the current study is the surface property of
23 ANTA film that is significantly different from carbon film. Using hPD-L1 as a test
24 sample, the single molecule force spectroscopy showed a strong none-specific
25 interaction between carbon film and the hPD-L1 protein. The measured force
26 (16.6 pN in average) is strong enough to absorb protein particles and induce
27 protein denature in a high probability, which might explain the non-uniform
28 distribution and aggregation effect of Hfn particles observed from cryoEM
29 micrographs. However, the measured force (0.94 pN in average) between ANTA
30 film and the hPD-L1 protein is less than 1 pN , which is very small and would not

1 induce significant change of the interacted particles. The strong non-specific
2 interaction between carbon film and protein sample would be due to the
3 hydrophobic nature of carbon film, which is easy to form hydrophobic interaction
4 with the hydrophobic surface of protein sample and also dangerous for protein
5 denature. While, as a metal film, the surface of ANTA film is hydrophilic, which is
6 favorable to reduce none-specific interaction with protein sample. In our many
7 applications (RyR1 sample in the present study), we found using carbon film it is
8 rare to allow the particles distribute in the hole while the case can be efficiently
9 solved by replacing to ANTA film. This observation would be due to the different
10 surface property between ANTA film and carbon film, yielding another advantage
11 of ANTA film.

12 It would be susceptible for the toxicity of ANTA film because of containing
13 nickel atoms. However, our experiments showed a good biocompatibility of ANTA
14 film suitable for cell growth. This observation is due to the formation of titanium
15 oxide layer on the alloy surface (Figueira et al., 2009), which blocks the release of
16 nickel ion and eliminates the potential negative effects on cell culture, yielding a
17 good corrosion resistance of ANTA film.

18 The potential ferromagnetic property of ANTA film is another concern for the
19 application of ANTA film. However, our measurement of magnetic moment of
20 ANTA film is much small compared to the strong magnetic field in the objective
21 lens and thus can be ignored. In addition, our cryoEM SPA experiments showed
22 we can achieve 2.4 Å resolution for Hfn sample using ANTA film. As a result, in the
23 resolution range of 2~3 Å, the potential magnetic effect from ANTA film is not
24 necessary to consider.

25 Overall, ANTA film provides another choice for cryoEM sample preparation
26 and has the potential to improve the sample distribution and increase the
27 efficiency of data collection in comparison with carbon film.

28 **DATA DEPOSITION**

29 The final cryoEM maps of human apo-ferritin from datasets using the grid
30 support of ANTA film and carbon film have been deposited into Electron

1 Microscope Data Bank (EMDB) with the accession codes EMD-XXXX and
2 EMD-XXXX, respectively. The corresponding datasets have been deposited into
3 EMPIAR with the accession codes EMPIAR-XXXX and EMPIAR-XXXX,
4 respectively.

5 **COMPETING INTERESTS**

6 Parts of this study (the preparation of ANTA supporting film with microarray
7 holes) has been submitted as a Chinese patent for invention with the application
8 number of 201810326897.5 and is currently under scrutiny.

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FIGURE LEGENDS

Figure 1. Preparation and usage of ANTA film. (A) Nickel titanium alloy wire is heated and evaporated under high vacuum to a fabricated silicon wafer with microarray holes. (B) The ANTA film on the silicon wafer is released on the water surface and transferred onto TEM grids (copper or gold). (C) A diagram to show how the ANTA film is attached onto one side of the TEM grid. The metal bar of the grid is colored in gold. (D) CryoEM images (low and high magnification) of ANTA film with human apo-ferritin vitrified on. The microarray hole pattern of ANTA film here is called R2-1 mix, which contains two kinds of hole sizes, 1 μm and 2 μm .

Figure 2. Thickness measurement of ANTA film by atomic force microscopy (AFM). (A) The topography image of ANTA film in a tapping-force mode. The film was transferred to a freshly exposed mica surface. (B) A zoom-in view of (A). The grey scale indicates the height. (C) The histogram of heights in (A). The two main peaks are fitted using Gaussian distribution. And the averaged thickness of ANTA film is measured as the distance between the two peaks. (D) The histogram of heights in (B).

Figure 3. Electrical resistivity and beam induced motion (BIM) of ANTA film in comparison with carbon film. (A) Electrical resistivity of ANTA and carbon film under a range of temperature from 77 to 300 K. The thickness of each film is prepared as 30 nm. (B) and (C) The in-plane BIM of ANTA and carbon film under different electron irradiation dose. The experiments were performed under 90K with human apo-ferritin vitrified on the film. The stage was tilted zero degree in (B) and 45 degrees in (C), respectively. Each data point represents the average displacement of the whole imaging area captured and the error bar shows the standard deviation of all measurements (see Russo and Passmore, 2014). RMS, root of mean square.

Figure 4. TEM characterization of ANTA film in comparison with polycrystalline gold and amorphous carbon films. The TEM micrographs of films (first column) were captured with the defocus of 3.5 μm and their power spectrums (third and fourth column) were measured with the defocus of 1.5 and

1 3.5 μm , respectively. The micrographs were taken at a magnification of 59,000x
2 with a total dose of 50 $\text{e}/\text{\AA}^2$. The electron diffraction patterns are shown in the
3 second column. The electron diffraction images were taken at a camera length of
4 850 mm with beam stop inserted and a total dose of 15 $\text{e}/\text{\AA}^2$.

5 **Figure 5. CryoEM SPA application of human apo-ferritin using ANTA film in**
6 **comparison with carbon film. (A) and (B)** CryoEM maps of human apo-ferritin
7 reconstructed from the datasets collected using ANTA film grid (A) and carbon film
8 grid (B), respectively. **(C)** Representative densities of both maps with atomic
9 models fitted. Left, is from (A) and right from (B). **(D)** The gold-standard FSC
10 (Fourier Shell Correlation) curves of both maps. **(E)** Henderson-Rosenthal plots
11 for both cryoEM SPA experiments.

12 **Figure 6. Particle distribution using ANTA film in comparison with carbon**
13 **film. (A) and (B)** CryoEM micrographs of 1.3 mg/ml human apo-ferritin frozen on
14 the same ANTA film grid were taken at both low (A) and high (B) distribution
15 density areas. **(C) and (D)** CryoEM micrographs of 1.3 mg/ml human apoferritin
16 frozen on the same carbon film grid were taken at both low (C) and high (D)
17 distribution density areas. **(E) and (F)** Typical cryoEM micrographs of 15 mg/ml
18 ryanodine receptor type 1 (RyR1) vitrified using ANTA film grid (E) and carbon grid
19 (F). More than 100 particles can be picked from (E) while less than 30 from (F).

20 **Figure 7. None-specific interaction between ANTA film and protein in**
21 **comparison with carbon film.** The statistics of rupture force interaction between
22 HPD-L1 and the supporting film was measured using AFM. The histograms of
23 force measurements are shown for ANTA film (A) and carbon film (B), respectively.
24 A Gaussian fit was performed to calculate the averaged none-specific interaction
25 force between HPD-L1 and the supporting film.

26 **Figure 8. Culturing hippocampal neuron cell on both ANTA film (A) and**
27 **carbon film (B).** Both films are coated onto gold grids.

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29

Figure 1

Figure 2

Figure 3

$\Delta f \sim -3.5 \text{ } \mu\text{m}$

diffraction

 $\Delta f \sim -1.5 \text{ } \mu\text{m}$ $\Delta f \sim -3.5 \text{ } \mu\text{m}$ Gold
film

C film

ANTA
film **Figure 4**

Figure 5

ANTA
grid

C
grid

1.3 mg/ml human apoferritin in
low distribution density area

1.3 mg/ml human apoferritin in
high distribution density area

15 mg/ml RyR1

Figure 6

A **B** **Figure 7**

ANTA film on gold grid

Amorphous carbon film on gold grid

Figure 8