Analysis of Egg White Protein Composition with Double Nanohole Optical Tweezers

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ABSTRACT: We use a double nanohole optical tweezer to analyze the protein composition of egg white through analysis of many individual protein trapping events. The proteins are grouped by mass based on two metrics: standard deviation of the trapping laser intensity fluctuations from the protein diffusion and the time constant of these fluctuations coming from the autocorrelation. Quantitative analysis is demonstrated for artificial samples, and then, the approach is applied to real egg white. The composition found from real egg white corresponds well to past reports using gel electrophoresis. This approach differs from past works by allowing for individual protein analysis in heterogeneous solutions without the need for denaturing, labeling, or tethering.

INTRODUCTION

The scientific community recognizes the need to develop new technologies for identifying and quantifying proteins in complex samples. For example, the US National Institute of Health noted: “our ability to identify and quantify proteins in complex (e.g., clinical) samples is progressing steadily, but it is clear that orders-of-magnitude advances in the associated technologies would enable substantial advances in a large range of biomedical research areas”.

There are many candidate technologies to achieve this goal, such as gel electrophoresis, mass spectrometry, labeling (e.g., fluorescence), and nanopores. Gel electrophoresis denatures the protein and removes it from its natural environment. Mass spectrometry also requires gas-phase ionization of the proteins. Labeling has been shown to alter the natural behavior of proteins. Although nanopores are intriguing for protein analysis, including shape and size, and even potentially sequencing, the fast translocation time makes analysis challenging and techniques such as tethering and optical tweezing have been suggested to slow down translocation.

Double nanohole (DNH) optical tweezers have been used to isolate single proteins in solution without the use of tethers or labels. This method does not denature the protein and allows for dynamic studies on the molecular level. It has shown signatures for protein–protein interactions, protein–small-molecule interactions, and protein–DNA interactions. The tweezers have also been used to obtain the low-frequency vibrational modes. In previous works, we have explored the dynamics of the trapping events and the particle escape from the trap due to Brownian motion. Here, we operate with higher laser power so there is no release once the particle is trapped. Occasionally, brief trapping events of a few seconds can occur, but those events were ignored in our analysis. In this work, we rely on the linear mass dependence of the fluctuations of the trapped laser signal and a −2/3 power mass dependence with its time constant to differentiate proteins based on mass in a heterogeneous mixture and in unpurified egg white.

These results show the promising capabilities of using DNH tweezers in other unprocessed physiological solutions, such as blood samples, to analyze trapped proteins and to translocate them for further reactions with different agents. Fabricating the DNH on the end of an optical fiber can be incorporated in a simple microwell setup, without any microscope optics. Multiple fibers can be used for simultaneously trapping molecules in the same sample or in different ones. Our technique has the potential of orders-of-magnitude improvement in the sensitivity of the analysis as it enables probing single molecules. Another significant improvement is in the reduced preparation time for dealing with raw samples, compared to other methods which require preparatory treatment such as purification.

METHODS

Figure 1a shows the optical tweezer setup modified from an optical tweezer kit (Thorlabs, OTKB/M) and force measurement model (Thorlabs, OTKBFM). The detector was replaced with a silicon-based avalanche photodetector (APD) (Thorlabs, APD110A). A half-wave plate (HWP) and polarizer were added to the setup to align the beam polarization to be perpendicular to the length of the DNH for maximum field enhancement. The trap laser was changed to a continuous laser which...
operates at a wavelength of 852 nm (Thorlabs, DBR852P). The laser power was maintained at 16 mW at the location of the trapping site.

DNHs were fabricated in a 100 nm thick gold film adhered to glass with a 5 nm Ti layer (EMF Corporation) using a focused ion beam (FIB) (Hitachi FB-2100 FIB & FEI Helios NanoLab 650) with a dwell time of 10 μs and 35 passes. The DNH was produced by milling two 180 nm circles with a 35 nm line separating the two circles. The resulting structure on the gold sample is slightly tapered throughout the thickness of the gold piece, which has been shown to increase the trapping efficiency. Figure 1b shows an image of the DNH, acquired using a scanning electron microscope.

The gold film containing the DNH was rinsed with isopropanol, methanol, and deionized water and dried using nitrogen gas. Samples were then prepared by pipetting 10 μL of sonicated solution placed between the gold film and a cover glass slide (Gold Seal, 24 × 60 mm no. 0). The different solutions used pure proteins (for reference ovalbumin, ovotransferrin, and ovomucoid—Sigma-Aldrich GE28-4038-41 & T2011-250MG) and chicken egg white from a local grocery store. All solutions were made in a phosphate buffer solution (1× PBS) and stored at 4 °C. The pure protein solution had a 1% concentration by weight, the control heterogeneous solution had equal parts of 1% ovotransferrin and ovalbumin solution by volume, and the egg white solution had a 1% concentration by volume. The gold film was adhered to the cover glass with adhesive microscope spacers (Grace Bio-Labs, GBL654002), which formed the microwell. The entire sample assembly was mounted in the inverted microscope optical trapping tweezer setup.

## ANALYSIS

Two specific properties were extracted from the trapped signals of each event, the standard deviation (SD) and the fast time constant (τ) of the autocorrelation function (ACF). Assuming a spherical shape, we express the trapping potential as proportional to the volume of the object (which scales as the radius cubed, \( r^3 \))

\[
U \propto r^3 \left( \frac{n_p^2 - n_m^2}{n_p^2 + 2n_m^2} \right)
\]

(1)

where \( n_p \) is the refractive index of the particle, \( n_m \) is the refractive index of the surrounding medium, \( r \) is the particle radius, and \( I_0 \) is the intensity of the laser beam at the trapping site.

The intensity of the transmission picked up by the APD is proportional to the potential energy so we can expect the signal intensity to scale with the molecule volume and the molecular mass (assuming a constant density)

\[
I \propto V \propto M_r
\]

(2)

The fluctuations in the signal intensity (Figure 2a) result from the Brownian motion of the particle in the trap. The motion of the particle in the trap can be modeled by an overdamped Langevin equation.

By taking the autocorrelation of the time series signal (Figure 2b), we can find its exponential decay time (\( \tau \)), which is used in conventional optical traps as a measure of the trapping stiffness

\[
\tau = \frac{\gamma}{\kappa}
\]

(3)

where \( \gamma \) is the Stokes’ drag and \( \kappa \) is the stiffness, which relates the gradient force to the displacement (\( \Delta x \)) of the molecule from the equilibrium position (like a spring constant).

\[
\tau = \frac{6\pi \eta r}{dF_{\text{grad}}/dx}
\]

(4)
In this work, the ACVF of trapped signals was found to fit well with a two-exponent function. We attribute the faster time scale change to be the one related to the stiffness of the trap. The slower time constant is assumed to be related to conformational changes of the proteins, as was found in previous works. We have shown in a previous work that the SD of the trapped signal has a linear dependence on the molecular mass of size standard proteins. We have also shown the time constant of the ACVF to have a $-2/3$ power dependence on molecular mass.

Figure 2a shows the time series of a typical trapping event, showing a distinction between the untrapped (blue) and trapped state (red). The inset shows a zoomed-in image of the signal variation in the trapped state that is used in this analysis. Trapping was observed and identified from the sudden increase in transmission observed on the APD. We have established previously that these are single-particle trapping events from the uniform size of the trapping step for a given particle and the distinct characteristic of (infrequent) multiple particle events.

Data from the APD were sampled at a rate of 100 kHz (Advantech USB-4711A) and were downsampled by a factor of 100 by straight averaging. The SD was calculated for a 5 s segment out of the trapped signal and was divided by the mean value of the untrapped signal (before the increase in the transmission signal).

Figure 2b shows the ACVF calculated for a 1 min segment of trapped data and fitted to a two-exponent function using the method of least squares. We attribute the slower time constant to conformational changes of the proteins that cause fluctuations in the trapped signal, as seen in our previous works. The faster time scale change is related to the stiffness of the trap. In this work, trapped egg white proteins were analyzed using two properties: the SD of the signal intensity (1) and the faster time constant ($\tau$). Using eqs 2 and 6, we approximate $\tau$ having a $-2/3$ power dependence on the signal SD.

## EXPERIMENTAL RESULTS

We first analyzed pure protein samples to ensure consistency of our setup with previous works. We then performed trapping analysis on a controlled heterogeneous mixture, a solution made of 50% ovalbumin and 50% ovotransferrin.

Figure 3 shows the SD as a function of $\tau$ for the individual trapping events in this artificial heterogeneous mixture. Two separate groupings are found, and using our analysis on the pure proteins, we attribute the black triangles to ovotransferrin and the blue circles to ovalbumin. Interestingly, an equal number of trapping events were found for each protein.

Figure 4 shows a similar analysis for egg white solution. A total of 25 events were obtained for this DNH, and the results of the SD and $\tau$ are plotted on a log–log scale. On the basis of SD and $\tau$, we attribute group A (black triangles) to proteins with a molecular mass higher than 49 kDa; trapping events in group B were classified as trapped particles with a molecular mass in the range of 36–49 kDa; and trapping events in group C were classified as trapped particles with a molecular weight lower than 39 kDa.

![Figure 3](image3.png) SD and ACVF time constant ($\tau$) of ovotransferrin and ovalbumin trapping events. A mixed solution of ovotransferrin and ovalbumin (1:1 ratio by volume).

![Figure 4](image4.png) SD and ACVF time constant ($\tau$) of egg white trapping events. SD of trapped signals with respect to their $\tau$. Trapping events in group A were classified as particles with a molecular weight higher than 49 kDa; trapping events in group B were classified as trapped particles with a molecular mass in the range of 36–49 kDa; and trapping events in group C were classified as trapped particles with a molecular weight lower than 39 kDa.

### DISCUSSION

We note that carbohydrates are also found in egg white but their abundance is lower than that of proteins. Their molecular weight is typically smaller (on the order of kilodaltons), and they are commonly bound to proteins influencing the protein molecular weight. It is unlikely that we can trap the carbohydrates alone, but proteins trapped with carbohydrates will have varying size because they appear as a complex. This may explain the larger variation seen in the time constants and SD of the egg white data (variation of 0.006 in egg white SD vs. 0.002 in pure ovalbumin SD and of 11 ms in egg white $\tau$ vs. 8 ms in pure ovalbumin $\tau$).

Table 1 compares the abundance (by $\tau$ and SD) of our trapping events with reports of egg white composition from the literature. The last column in the table does not include proteins that are less than 1.5% in composition or have a very small abundance.
large molecular weight (which may be too large to enter the trapping site). Considering the statistical error, good agreement is seen between our trapping method and those past results.

Fouling of the DNHs occurs after a few days of continuous measurement, which limits the collection period. Figure 5 shows the scanning electron microscopy (SEM) images of a new DNH aperture just after it has been milled, compared with an aperture that was used for continuous trapping over a week. The fouling of the DNH structure is seen. The optical signal does not show trapping on these fouled samples. We fabricate several DNHs on a single gold sample, and each of these can be used in series; however, there is some signal variation between the DNHs (which is in part due to the grains in gold and the instabilities in the FIB).

The time to trap is quite long in our samples. At 0.01% pure sample, the time to trap was prohibitively long. At 0.1 and 1%, the trapping times were similar (on the order of minutes). On the basis of diffusion considerations alone, we would expect a much faster time to trap; however, for these small particles, we suspect that surface-charge effects are playing a role, as recent ionic detection work has noted that screening around metals does limit detection events until very high concentrations are seen. The surface charge on the glass is negative. Because of the phosphate buffer, there also exists a net negative charge on the proteins. The like-charges repel the proteins, which results in a longer time to trap. We are presently investigating different surfaces to attempt to mitigate this effect and obtain higher throughput trapping. For example, applying a monolayer of methoxypoly(ethylene glycol) (mPEG) thiol was used to reduce effects of protein absorption into the gold sample. The optical signal will get affected in case of protein adsorption within the nanoaperture itself which makes the DNH not useful for trapping, in which case, a different one must be used. We confirm that single trapping events are not the result of particles adsorbed to the surface by blocking the laser after each event and observing the voltage level returning to the untrapped level prior to the next trapping event, as shown in Figure 6. Figure 7 shows a second jump (increase in transmission) within the trapped state. We attribute that to a second molecule that enters the trap, as was shown in our previous work. These rare events were not studied in this work and have been excluded from the data collected.

The slope of the fit of Figure 4 is approximately -0.66, in good agreement of the scaling of mass to the -2/3 power to τ and linearity to SD. Previously, we obtained anomalously large τ values for ovotransferrin, which have been modified to be in line with theory by using the two-time constant fit. The appearance of two time scales (perhaps the slower being from conformational/orientational changes of the protein) was clear in the time series data.

Table 1. Classification of Egg White Protein Composition

| group | M_r range (kDa) | number of events | percentage (%) |
|-------|----------------|-----------------|----------------|
| A     | M_r < 49       | 2               | 8              |
| B     | 36 < M_r < 49  | 19              | 76             |
| C     | M_r < 36       | 4               | 16             |

| protein name | M_r (kDa) | abundance in egg white (%) | scaled abundance in egg white (%) |
|--------------|-----------|-----------------------------|----------------------------------|
| ovotransferrin | 77.7      | 12                          | 13.6                             |
| ovoglobulin G3 | 49.0      | 4                           | 62                               |
| ovalbumin    | 44.5      | 54                          |                                  |
| ovoglobulin G2 | 36.0      | 4                           |                                  |
| ovomucoid    | 28.0      | 11                          | 14.4                             |
| lysozyme     | 14.3      | 3.4                         |                                  |

a) Left: grouping of experimental data (as shown in Figure 4) and classification by molecular weight (M_r). Right: main proteins found in egg white. b) Neglecting proteins with low concentrations (≤1.5%) and those >500 kD.
The right side of Figure 8 shows the time traces and ACFs of single trapping events out of egg white proteins that were characterized in each one of the groups (A, B, and C in Figure 4). A comparison can be made with pure protein trapping events (ovotransferrin, ovalbumin, and ovomucoid) that are presented on the left of Figure 8. Overall, inspection of the trapping events with the pure proteins and the egg white proteins shows matching characteristics. Slight variations in the signal were observed even for the same protein; however, these were also present for the pure proteins, so we attribute them either to variations in the trapping landscape or natural variations in the protein. Nevertheless, the SD amplitude and time constant analysis gave similar values among the same protein.

CONCLUSIONS

We have demonstrated the use of DNH optical tweezing in the analysis of protein composition of unpurified heterogeneous solutions. This development allows for single-molecule control, enabling studies on isolated proteins for analysis of their dynamics and interactions. For example, our method can differentiate mutant and wild-type p53 from characteristics with DNA interactions, and drug developments such as correcting mutant forms of proteins like metallochaperone for p53 will benefit from single-molecule control. Also, compositional analysis can be performed on food and other mixtures. On the basis of scaling-up to several fibers (about 10) and reducing the trapping time to below a minute, analysis of around 1000 proteins per sample is envisionable in the future. This method is unlikely to compete with high throughput methods but does have advantages of providing single-molecule analysis such as allowing for richer statistics and interacting the isolated species with other entities. With these improvements, this method is promising for disease diagnosis, drug development, targeted therapy, food analysis, and basic protein studies in solution.

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Figure 7. Time trace of a dual trapping event. A second jump in the laser transmission after the first trapping event. We assume this is a result of a second molecule entering the trap.

Figure 8. Time traces and ACFs of pure protein and egg white protein trapping events. (a) Trapped ovotransferrin; ACF is fitted to $1.19e^{-t/4.69} + 0.01e^{-t/1380}$. (b) Trapped egg white protein from group A; ACF is fitted to $1.19e^{-t/3.91} + 0.06e^{-t/1380}$. (c) Trapped ovalbumin; ACF is fitted to $0.9e^{-t/6.85} + 0.17e^{-t/108}$. (d) Trapped egg white protein from group B; ACF is fitted to $0.79e^{-t/9.87} + 0.29e^{-t/71.3}$. (e) Trapped ovomucoid; ACF is fitted to $0.7e^{-t/23.36} + 0.3e^{-t/186}$. (f) Trapped egg white protein from group C; ACF is fitted to $0.58e^{-t/21.75} + 0.37e^{-t/456}$. Groups A, B, & C are indicated in Figure 4. Time constants are given in milliseconds.
Author Contributions
N.H. performed the experiments. N.H., C.J.X.I., and R.G. performed the analysis and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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