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Label-Free Free-Solution Single-Molecule Protein–Small Molecule Interaction Observed by Double-Nanohole Plasmonic Trapping

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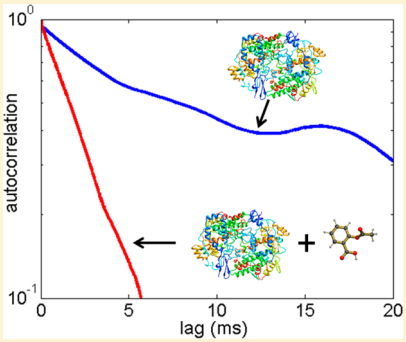
ABSTRACT: The interaction of proteins with small molecules is fundamental to their function in living organisms, and it is widely studied in drug development. Here we use the double nanohole optical trapping technique to observe real-time label-free free-solution single-molecule dynamics of three complexes: biotin–streptavidin, biotin–monovalent streptavidin, and acetylsalicylic acid–cyclooxygenase 2. Radically different behavior is seen between the protein with and without the small molecule binding. This detection platform is scalable, inexpensive, and highly sensitive, which may transform drug discovery based on protein–small molecule interactions.



KEYWORDS: *double nanohole, optical trapping, nanoparticles, nanoplasmonics, subwavelength apertures*

Protein–small molecule interactions (PSMIs) play an important role in biological functions. PSMIs are also of primary interest for the development of drugs, for example, through inhibition of protein interactions.[1] While many works have studied PSMIs, only a few approaches exist that do not require tethering to a surface or labeling. Tethering to a surface has the disadvantages of using a binding site, restricting the protein motion with an anchor, and introducing steric hindrance from the surface proximity. Exogeneous labels, such as fluorescent tags, present similar challenges, including using up a binding site and altering the natural state of the molecules of interest, but also add the complexity of using a label. So far, calorimetry[2] and interferometry[3] have been used as label-free, free-solution techniques. Calorimetry has high concentration detection limits and is restricted to systems with an appreciable reaction enthalpy. Interferometry makes use of refractive index changes from PSMIs and can detect PSMIs in the micromolar range.

Ideally, we would like to introduce new label-free, free-solution methods that work at the single-molecule level, where it is possible to observe real-time dynamics that are not obscured by an ensemble. There are many advantages of working at the single-molecule level.[4,5] For example, with access to these dynamics and without the need for synchronization, we can compare more directly to molecular dynamics calculations (for example, ref 6). Working at the single-molecule level represents the ultimate practical sensitivity limit. It also offers opportunities for distinguishing components of heterogeneous systems, such as cell lysates.

Here we consider the use of optical trapping to observe, in real time, the dynamics of a single streptavidin molecule, comparing the cases with and without exposure to biotin. Our optical trapping approach is similar to that reported in previous works,[7−11] as shown schematically in Figure 1a. Briefly, a double-nanohole aperture is milled using a focused ion beam in a 100 nm Au film adhered to a glass slide with a 5 nm Ti layer (Figure 1c). The gold film forms the top of a microwell in an inverted microscope optical trapping setup using an 820 nm laser diode. The transmission of the laser diode through the double-nanohole (DNH) aperture is used to detect and monitor the trapping events since dielectric loading creates a large variation in the transmitted intensity. Typically, a trapping event gives a 10% change in transmission, depending on the size of the nanoparticle trapped and the size of the aperture.[8] Our previous studies show that trapping of two particles of a comparable size induces a two-step-increased transmission through the DNH structure,[12] and this has also been reported by other groups (for example, ref 13). We have not observed such behavior in our current study, which is likely due to steric hindrance, which prevents trapping of two similar particles between the two sharp tips of the DNH.[8] It should be noted that this method produces negligible heating due to the presence of a gold film; the heating is expected to be on the order of 0.1 K.[14,15] Furthermore, the technique produces copious signal for only 3 mW of laser power, such that an optical density filter is used to avoid saturation of the avalanche photodiode (APD).

We prepared streptavidin solutions (Sigma-Aldrich, 85878, molecular weight 60 kDa) in buffer with 0.01% w/v concentration. A portion of the solution was separated and exposed to excess biotin (Sigma-Aldrich, B4501, molecular weight 244.31 Da), which fully saturates the binding sites due to its high affinity. Figure 2 shows the trapping dynamics of the streptavidin solution with and without the biotin. For the concentrations used in this study, the time to trap was typically

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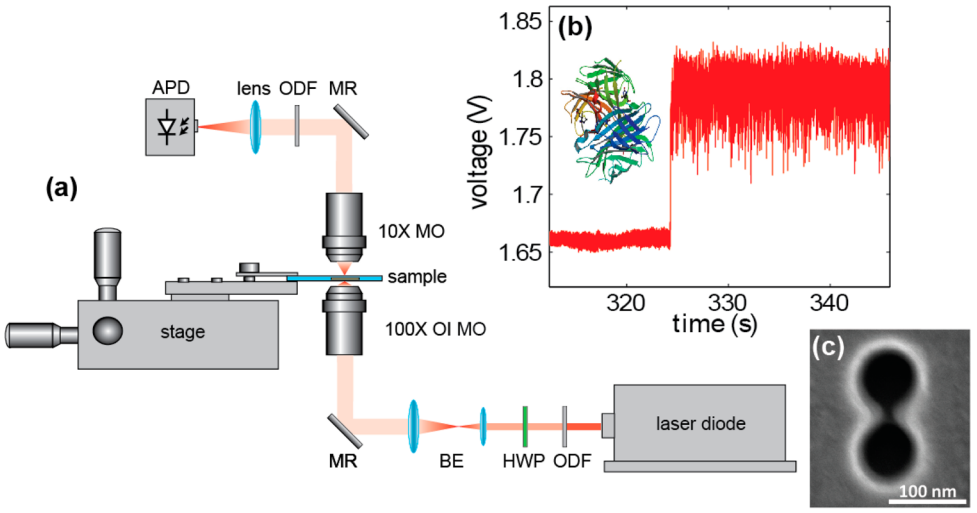

Figure 1. (a) Schematic of the double-nanohole optical trap. Abbreviations used: ODF = optical density filter; HWP = half-wave plate; BE = beam expander; MR = mirror; MO = microscope objective; OI MO = oil immersion objective; APD = avalanche photodetector. (b) Optical trapping of biotinylated streptavidin (inset) seen as a sudden discrete jump in APD signal. (c) SEM image of the double-nanohole.

5 min; however, this can be made shorter by increasing the concentration, and we have studied this quantitatively in another work.[10] The trapping event is seen by a discrete jump in the transmitted laser intensity, as denoted by arrows in Figure 2a and d. The streptavidin without biotin shows fluctuations in the transmitted intensity of the trapping laser with a time scale of about 600 ms, as seen in Figure 2b and c (taken from different samples on different days). These fluctuations are absent in the biotinylated streptavidin, as seen in Figure 2e and f. An autocorrelation of the trapping events found for streptavidin and biotinylated streptavidin is shown in Figure 3, which shows clearly the slower time scale dynamics of the streptavidin as compared to the biotinylated streptavidin molecule. We preformed these experiments on different days and from different solutions, and all the results obtained are consistent with Figure 3. To obtain the autocorrelation, we typically used a time sequence of 400 s (sampling at 1 MHz) after trapping. This extended time duration is highly conservative, but in general, at least 100 periods of the shortest time scale of interest are recommended to avoid spurious artifacts.[16] We then used the Matlab function autocorr() to compute the autocorrelation.

The change in the light transmitted through the DNH aperture can arise from differences in the size of the particle trapped,[10] but also from changes in the particle shape or orientation, for example due to unfolding of proteins[9] or conformal changes. These marked differences are not expected to arise from mass loading since biotin has a mass <0.5% of the mass of streptavidin. The streptavidin without biotin shows fluctuations that are not present when biotin is added. This is consistent with numerical studies of streptavidin that suggest that the binding loop is highly mobile in the absence of biotin;[17,18] it would be interesting to attempt quantitative comparisons with molecular dynamics simulations in the future. There are many cases in the literature where binding of a small molecule alters the molecular dynamics of a protein substantially (for example refs 19 and 20). The change in the optical transmission through the double-nanohole aperture is expected to vary considerably due to stretching of the protein since the dipole moment increases along the axis of elongation. It is energetically favorable of the optical trap to stretch out the protein, and this leads to more light transmitted through the DNH. Similar stretching behavior has been reported in other optical trapping systems, albeit for much larger particles such as cells.[21] The increase in intensity at the trapping event comes from dielectric loading of the DNH by the protein, which allows for more light transmission. The trapped particle is subject to Brownian motion and conformal changes, which also change the amount of light transmitted and show up as increased fluctuations in the signal after trapping. A more elongated particle should have higher polarizability and, therefore, will give higher light transmission through the aperture. The streptavidin alone has larger fluctuations than the streptavidin−biotin complex, and these suggest that the streptavidin can fluctuate between an elongated and compressed state in the absence of biotin. These fluctuations occur on a time scale of 600 ms, which is directly related to the autocorrelation as the slope of the decay. The fluctuations of the biotinylated streptavidin are likely to be from translational Brownian motion and have a time scale of 20 ms, as we have seen for the autocorrelation of nanoparticles in our past work.[11] In our previous study, the DNH unfolded bovine serum albumin (BSA),[9] which is well known to have an elongated unfolded state.[22] It appears that the DNH does elongate the streptavidin, as seen by increased light transmission through the aperture, which comes from high polarizability of an elongated streptavidin molecule; however, we have not found any reference to the hinge-like unfolding of streptavidin as seen with BSA. Figure 2d shows occasional steps for the biotinylated streptavidin, which suggests that conformal or orientation changes can occur even in the presence of biotin, although much less frequently as compared with bare streptavidin. We do not believe that these changes are the result of dissociation of the biotin, since this typically occurs on the time scale of 10^8 s.[23] Further investigation, perhaps comparing with numerical simulations, should help to clarify which process is taking place here. In addition, the autocorrelation approach may be used to measure transitions between the bound and unbound states and extract binding kinetics; however, this was not seen in the present work due to the high binding affinity of the biotin−streptavidin system. The confidence of distinguishing the bound and unbound states will require typically autocorrelation

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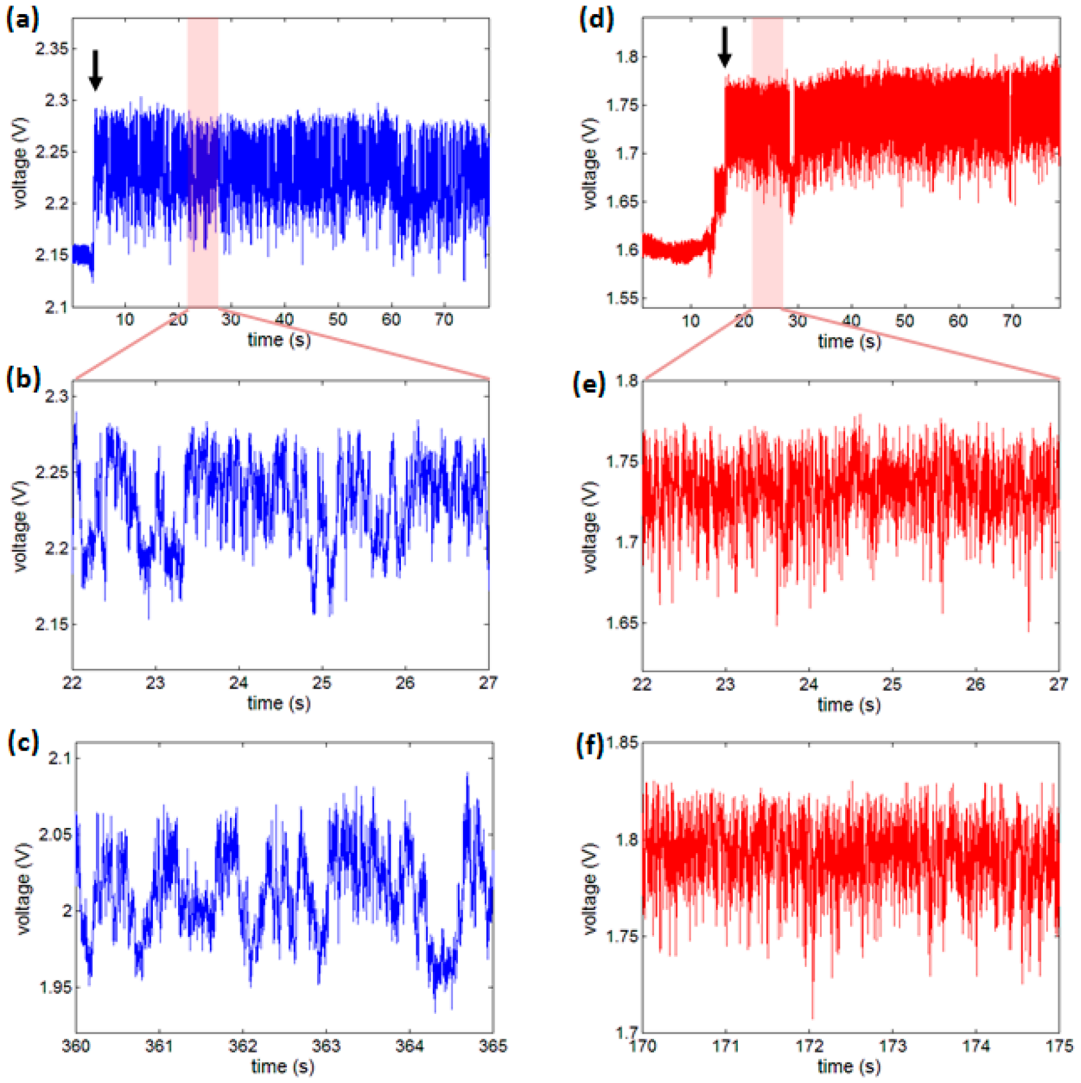

Figure 2. Trapping dynamics of streptavidin without and with biotin as measured from the APD voltage. (a) Time trace of a trapping event of a bare streptavidin molecule seen as an abrupt jump in the voltage level as denoted by the arrow. (b) Zoom-in of (a). (c) Repeat of (a) taken from a different sample on a different day. (d) Time trace of a trapping event of a biotinylated streptavidin molecule seen as a discrete jump in the voltage level as indicated by the arrow. (e) Zoom-in of (d). (f) Repeat of (e) taken from a different sample on a different day.

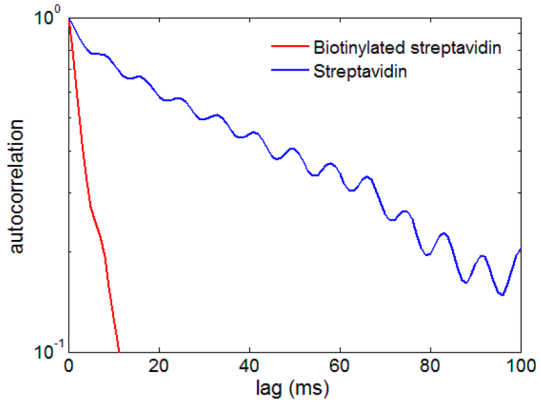

Figure 3. Autocorrelation of trapped streptavidin APD signal fluctuations with and without biotin, as seen in Figure 2b and e.

times that are 100 times the shortest time scale of interest, as mentioned previously. In the present study, the time scales of interest are around 10 to 100 ms, so this would limit the autocorrelation to the study of binding rates on the order of 0.1−1 Hz. This is also clear from Figure 2b and c, even without the autocorrelation, where the "jumpy" behavior has a time scale on the order of 1 s, and so we can see transitions between the two states by analysis of the time series as well (e.g., by looking at the RMS deviation). We have observed fouling of the nanoholes as seen by a drop in intensity, which depends on the cleanliness of the Au sample handling. When this happens, we no longer use the trap, and we have not studied the time dynamics of this process. With care, the typical period of usage for a single sample is around a month. A particle of comparable size to the proteins being studied would also show a step-like time response, but would give a different time series (e.g., autocorrelation would vary), as we have studied in detail in other works.[10,11]

To further establish the applicability of our approach to distinguish between the bound and unbound forms of a protein, we used two different proteins: (i) monovalent streptavidin and (ii) cyclooxegenase 2. As for (i) we obtained monovalent streptavidin (molecular weight 54 kDa) with an E6 tag as described in ref 24 from the Howarth group, UK. The monovalent streptavidin solution was prepared at 0.01% w/v

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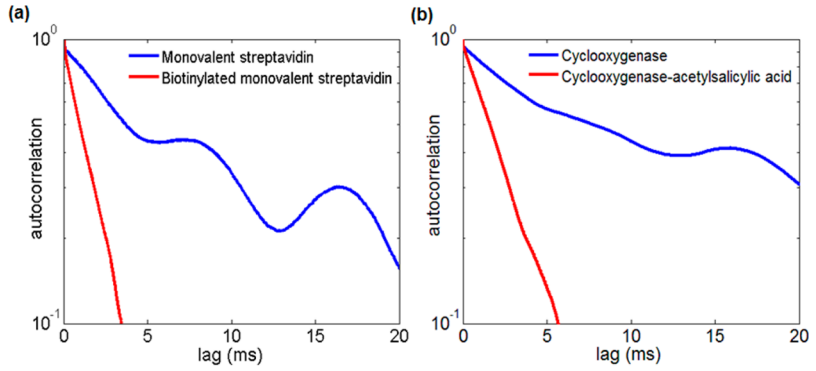

Figure 4. Autocorrelation of time traces of trapped monovalent streptavidin (a) and cyclooxygenase 2 (b) with and without small molecule binding.

concentration in buffer with some portion of it mixed with excess biotin to block the active binding site of the monovalent streptavidin. For (ii) we prepared cyclooxygenase 2 (Sigma-Aldrich, C0858, molecular weight 72 kDa) in buffer with 0.01% w/v concentration. A portion of the cyclooxygenase 2 solution was mixed with acetylsalicylic acid (known also as asprin) with a molecular weight of 180.16 Da, which irreversibly inhibits the cyclooxygenase by binding the acetyl group of acetylsalicylic acid to a serine residue of cyclooxegenase protein.[20] Figure 4a and b show the respective autocorrelation of the time traces of a trapped monovalent streptavidin and cyclooxegenase proteins with and without the small molecule binding. It is clear from both figures that a bare form of the protein in the trap has a slower time variation as when compared with the bound protein, which can be easily inferred by looking at the autocorrelation of the signals in Figure 4a and b.

Future measurements are anticipated to observe the individual biotin-binding events. In order to achieve this, we are considering a flow-channel setup, similar to our past work using nanospheres,[15] which may be used to first trap the vacant streptavidin and then introduce biotin into the channel. The goal here will be to determine if the binding to the four binding sites in streptavidin can be observed individually. Obviously, it is of great interest to see if these results can be further extended to other PSMI systems that play a role in biological function. Also of interest is to see how scalable this approach is to enable multiplexed screening, for example by using multiple optical traps.[25−27]

In summary, we have shown that by studying the optical trapping dynamics on a single protein we can easily distinguish between the bare and bound forms of a protein. Our approach does not require surface immobilization or exogeneous markers, and it gives the real-time dynamics of individual protein molecules, representing the ultimate practical limit for sensitivity. In addition, this work shows that our approach has a great potential for applications to screening small molecule drug candidates by monitoring their influence on proteins of interest[1] and for understanding the mechanisms of PSMIs.[28]

METHODS

Fabrication of DNH. We used a Hitachi FB-2100 focused ion beam to mill the DNH in a 100 nm thick Au film on a glass substrate with a 5 nm Ti adhesion layer (EMF Corporation). The structure was fabricated at a magnification of 80 k with the accelerating voltage and current of the gallium ion beam being 40 kV and 0.001 nA, respectively.

Gold Sample Preparation. A gold film sample, which contains the DNH as described in a past work,[8] was washed with acetone, rinsed with 2-propanol, and dried with nitrogen. An imaging spacer (Sigma-Aldrich, GBL654002) is placed on cover glass no. 0 (Goldseal, Ted Pella, Inc.), which forms the microwell. A 150 μL amount of the protein solution was pipetted into the chamber, and then the gold sample (with the gold side facing down) was placed on the imaging spacer to seal the well. The whole sample assembly was then mounted in the inverted microscope optical trapping setup as shown in Figure 1a.

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Notes

The authors declare no competing financial interest.

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