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Conformational Dynamics of a Single Protein Monitored for 24 h at Video Rate

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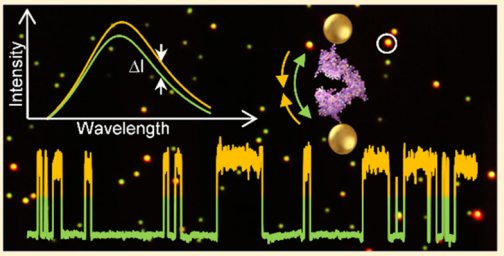
Supporting Information

ABSTRACT: We use plasmon rulers to follow the conformational dynamics of a single protein for up to 24 h at a video rate. The plasmon ruler consists of two gold nanospheres connected by a single protein linker. In our experiment, we follow the dynamics of the molecular chaperone heat shock protein 90 (Hsp90), which is known to show "open" and "closed" conformations. Our measurements confirm the previously known conformational dynamics with transition times in the second to minute time scale and reveals new dynamics on the time scale of minutes to hours. Plasmon rulers thus extend the observation bandwidth 3−4 orders of magnitude with respect to single-molecule fluorescence resonance energy transfer and enable the study of molecular dynamics with unprecedented precision.



KEYWORDS: *Plasmon ruler, single molecule, Hsp90, protein dynamics, nonergodicity*

The function of proteins is determined by a combination of their structure and dynamics. The acquisition of structural information has recently been revolutionized by advances in electron microscopy;[1,2] however, protein dynamics are difficult to obtain over long time scales.[3] Here we show a novel plasmon ruler based single-molecule approach to study the conformational dynamics of single proteins over 24 h at video rate in and out of equilibrium (i.e., in the absence and presence of ATP). This approach does not impose external force on the proteins and allows us to measure protein dynamics over several orders of magnitude, which cannot be achieved by the established single-molecule techniques.[4−6] We used this technique to explore the dynamics of the heat shock protein 90 (Hsp90) and find (beside the well-known fast dynamics in the 0.1−10 s range) states with very long dwell times (linked to rarely visited states) in the minute time scale, which could indicate, for instance, pathways to protein denaturation. Our plasmon ruler based method will enable us to access the full complexity of correlated local and global conformational dynamics on the level of individual proteins and therefore extend and complement the current "structure−function" paradigm with a novel "structure−time scale−function" description. Access to the dynamics of a single protein over more than 6 orders of magnitude further allows us to address important questions like conformational heterogeneity among proteins, ergodic behavior, and non-Markovian dynamics, ultimately extending the often static view of biomolecules with a more-dynamic picture.

Light induces the collective oscillation of the conduction electrons in noble metal nanoparticles with a specific resonance frequency called particle plasmon.[7] A plasmon ruler consists of two plasmonic (e.g., gold) nanoparticles bridged by the macromolecule under investigation (Figure 1a inset) (see the Supporting Information and Figures S1 and S2 for the nanoparticle preparation, characterization, and optimization of the assembly). The coupling of the plasmons of the two nanoparticles depends strongly on their separation: decreasing the interparticle distance shifts the plasmon resonance to higher wavelengths ("red shift") with an additional increase of the scattering efficiency (Figures 1a and S3). By continuously monitoring the scattering intensity of hundreds of single-particle pairs (plasmon rulers) in parallel under a dark-field microscope, we determine the interparticle distance and, thus, reveal the dynamics of the bridging macromolecule[8,9] (Figures 1a and S3). Plasmonic nanoparticles offer the possibility to access a wide range of time scales because of their unlimited photostability and exceptionally strong light scattering. These properties allow us to measure for, in principle, infinite time

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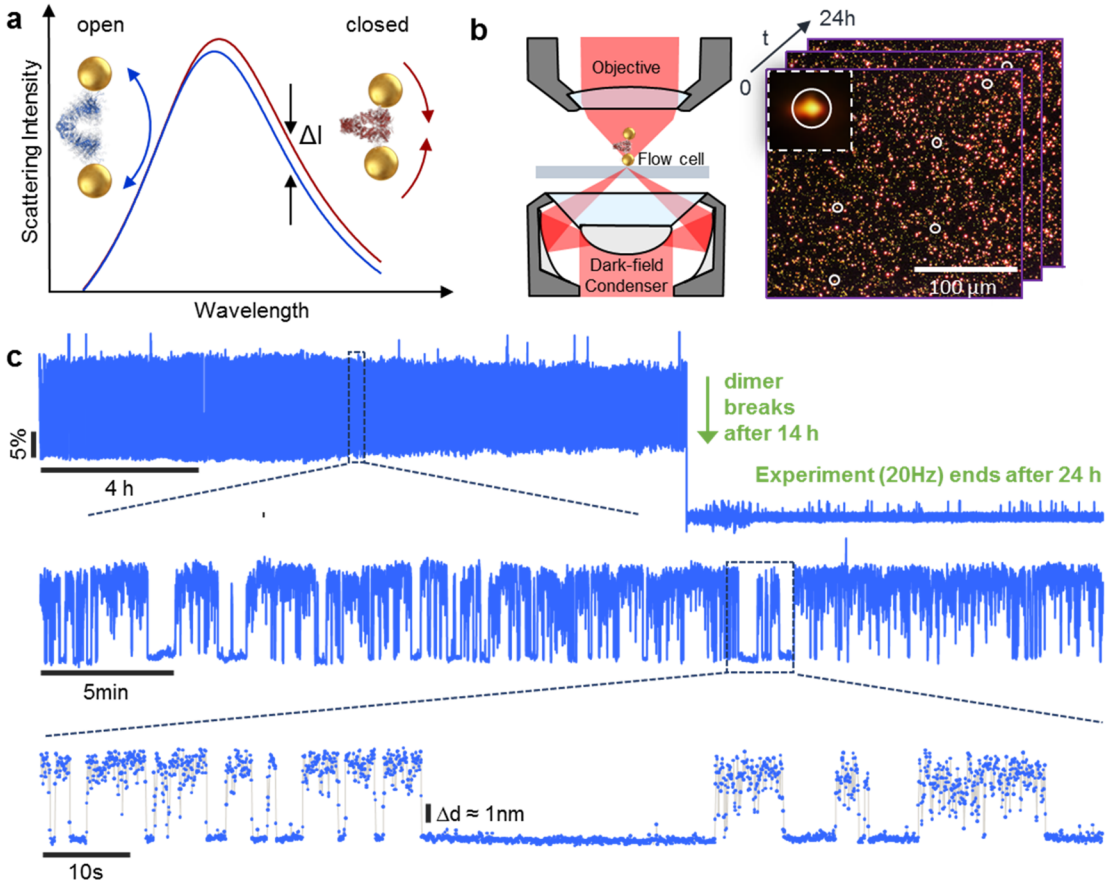

Figure 1. Plasmon rulers' time traces show Hsp90 dynamics on a time scale of milliseconds to hours. (a) Change of interparticle distance leads to shift in scattering spectra and thus allows us to distinguish the open (blue line) and closed (red) configuration of an Hsp90 sandwiched between two plasmonic nanoparticles. Both overall intensity and the intensity at a given wavelength (black arrows) changes with interparticle distance. (b) Many Hsp90 linked plasmon rulers can be observed in parallel. Under a dark-field microscope (left side), the scattered light from the nanoparticles can be collected with low background noise (right side). We follow the intensity of those dots over time (examples indicated by the white circles). (c) The relative intensity (normalized to its mean) of a single plasmon ruler (blue line) as a function of time. In this example, the plasmon ruler was measured every 50 ms (at 20 Hz) for 24 h. After about 14 h, the dimer breaks in a single step (confirming that only one linker connected the dimer initially). Dynamics can be observed at time scales ranging from hours to milliseconds. We show a zoomed-in section depicting 40 min of the fluctuating part and another zoomed-in section depicting 2 min (indicated by the black dashed line). The zoomed-in section at the bottom shows individual data points (blue circles) connected by gray lines as a guide to the eye.

with microsecond time resolution (see the Supporting Information for a discussion of the limits of plasmon ruler dynamical measurements and Figures S4–S6 and Tables S1 and S2). Plasmon rulers have been used to study DNA molecules and their interactions with proteins[10−13] and simple polymers[14] and can even be extended to study three-dimensional motions.[15] Until now, plasmon rulers have not been used to study conformational dynamics of more complex macromolecules such as proteins. Here, we report key technical and methodological advances in time resolution, nanoparticle functionalization, and measurement parallelization that permitted us to follow the dynamics of single proteins for up to 24 h at video rate (20 Hz) (Figures 1b and S7). Figure 1c shows an exemplary long time trace from a single molecule. From this single time trace, a complete dynamic state model and its transition rates can be deduced. Previously, experiments with single-molecule fluorescence resonance energy transfer (FRET) yielded time traces of around 100 s, requiring the pooling of hundreds of single protein traces to extract transition rates. Pooling of traces from different molecules implies ergodicity and the absence of additional dwell times on time scales more than about a factor of 2 larger than the trace length. This study on the molecular chaperone Hsp90 shows that these assumptions have to be revisited.

We use the Hsp90 as our model system because it has been extensively studied by FRET, X-ray crystallography, and electron microscopy.[16−19] The Hsp90 protein forms homodimers, stably connected through the two C-terminal domains and temporarily through the N-terminal domains. The protein complex undergoes scissor-like conformational changes in which the N-terminus is "open" or "closed".[16] We sandwiched a single Hsp90 complex between two gold nanospheres in a microfluidic flow cell. In our deposition strategy, one gold nanoparticle serving as anchor was attached to the glass slide. Then, a second particle was attached to the anchor particle with an Hsp90 complex bridging the two as schematically represented in the inset of Figure 1a. We use polyethylene glycol (PEG) for the passivation of the flow cell surface and the nanoparticles, as well as molecular linker for the protein-nanoparticle connection (see details and controls in the Figures S2, S4, S5, and S6). Our assembly strategy results in plasmon rulers linked by a single functioning Hsp90 complex.

Some plasmon rulers in the field of view (usually around 10%) show the telltale signature of the Hsp90 complex known

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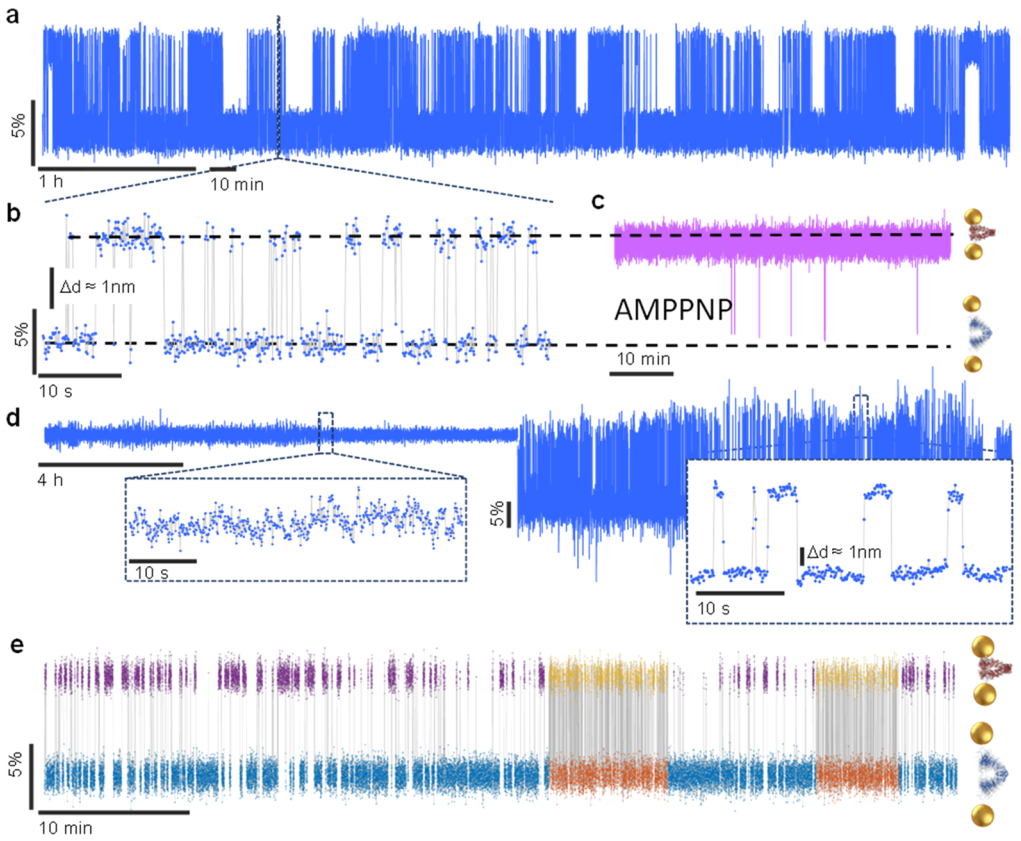

Figure 2. Nucleotide-dependent single-molecule time traces of Hsp90. (a) Example of a time trace of Hsp90 transitions between open and closed states recorded for 6 h at a time resolution of 100 ms. (b) Zoomed-in section depicting the time trace shown in panel a, as indicated by the dashed lines. The blue dots are the actual data points, connected by gray lines as a guide to the eye. The open and closed state are clearly separated in relative intensity. The relative intensity can be roughly converted to separation distance Δd in nanometers as indicated by the second vertical scale bar. (c) The same plasmon ruler is locked in its closed state for many minutes after addition of AMP-PNP to the buffer (pink line), which indicates a functional protein. (d) Another example of an Hsp90 time trace recorded for 24 h at 20 Hz. This example shows the situation in which Hsp90 is "stuck" in its closed conformation for the initial 12 h before resuming rapid transitions between the open and closed state. Insets show zoomed-in views of both parts. (e) Example of a part of a time trace in which the data points are colored according to the most likely substates, as determined by a hidden Markov analysis with four states.

from FRET experiments:[16,19] two distinct conformations (open and closed) with transitions in the second time scale (see Figures 1c and 2a,b). The mean distance change (4.7 ± 0.4 nm) agrees well with the value of 5.2 nm expected from available structures for this mutant (285C-285C), which reads 14.1 nm in the open state[20] and 8.9 nm in the closed state.[21] As an additional control, we add the nonhydrolyzable ATP analogue AMP-PNP at the end of the experiment, which is known to lock the protein in the closed state,[19] which we also observe here (Figure 2c). All of these observations make us confident that the plasmon rulers indeed capture the conformational dynamics of a single Hsp90 dimer. We excluded any influence from the gold nanoparticles on the natural conformational dynamics of Hsp90. First, for 60 nm gold spheres, the diffusion time is around 0.1 μs for a distance change of 1 nm. This diffusion time is around 6 orders of magnitude faster than the faster Hsp90 dynamics, which is around 0.1 s. We assume that it is possible to measure protein's conformational dynamics slower than this diffusion time. Second, the Hsp90 mutant was selected to avoid any interference or steric hindrance with the gold spheres. Third, the obtained Hsp90 dynamics from the plasmon rulers is in good agreement with the FRET measurements (in the 1 to 100 s range).

However, the plasmon ruler traces show dynamics never seen before by FRET: dynamics on the 1−10 min time scale (Figure 2a) and even molecules that remain in either the open or the closed configuration for several hours (Figures 2d and S7). Such long-lived states were not observed for plasmon rulers connected only by PEG linkers (Figure S5). We compare these plasmon ruler traces (outside of the regions where they remain in one state for extended period of time) to previous FRET studies using an analysis based on a hidden Markov model with four states.[22] Figure 2e depicts the four states from the Viterbi path in different colors. In general, the rates extracted from the plasmon rulers agree well with the FRET data (Figure S8). What is missing from the FRET data are long-lived states. We first quantify states with an intermediate occupancy in the minute time scale. For this analysis, we selected traces (or parts of traces) that are not "stuck" in one state for hours and converted them into a series of dwell times corresponding to the time spent in the open and closed conformation. In total, we obtained 27 700 transitions in the absence of ATP and 33 000 transitions in the presence of ATP (Figure 3). The cumulative occurrence, $P(t \geq \tau)$, data show linear regions on a negative logarithmic axis for dwell times in the hundreds of second region, which indicates another long-lived sub-state within the open and closed conformation. The lifetimes of these previously unknown

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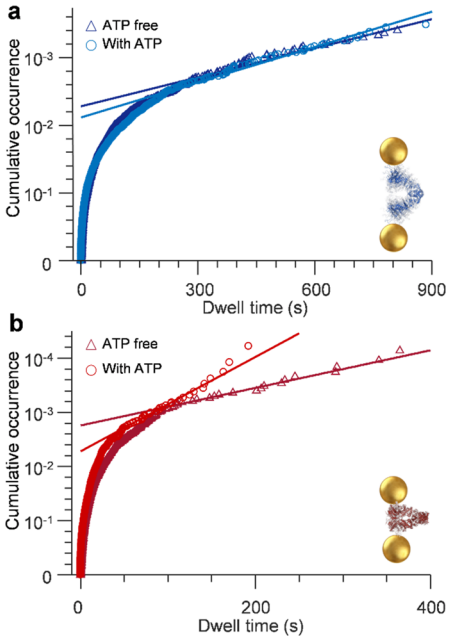

Figure 3. Quantification of previously inaccessible slow dynamics. The cumulative dwell time distribution $P(\tau)$ gives the probability to find dwell times shorter than τ. To make rare states with long lifetimes more visible, we display the cumulative occurrence $P(t \geq \tau)$ on a (negative) logarithmic axis, in which a statistically independent process is represented as a straight line. The triangles correspond to an ATP-free buffer, and the circles correspond to an experiment in the presence of ATP. (a) The cumulative occurrence for the open configuration. (b) The cumulative occurrence for the closed configuration. In both cases, there is a linear region with slopes corresponding to dwell times in the 100 s of second regime (blue and red lines). This long-lived state is significantly affected by ATP in the closed configuration.

states are between 4 and 5 min for the open conformation and one to 2 min for the closed conformation, with ATP reducing the lifetime in the closed conformation (Table S3).

These unexpected long-lived states, especially those on the hour time scale, clearly show the need to observe single molecules for extremely long periods: in this system, even 24 h are not sufficient to ensure ergodicity.[23] In an ergodic system, statistical properties from transitions within a single-molecule trace or from the same amount of transitions from different molecules yield the same result; however, we still find differences (for example of the total time spend in the open configuration) between 24 h traces (Figures S7 and S9). Ergodicity is an assumption generally taken in the single-molecule field,[24] which needs to be revisited in the light of these results. The long-lived states could correspond to misfolded protein domains. This would fit well with the findings from optical tweezer experiments that domain unfolding at zero force occurs on the time scale of hours and that the generally very fast refolding is often hindered by long-lived misfolded states for the full-length multi-domain Hsp90 protein.[25,26]

The analysis of these transitions within the Hsp90 molecule shows the capability of plasmon rulers to find conformational dynamics at both fast and slow time scales from one single protein complex. The accessible bandwidth is paralleled only by electrophysiological measurements on single ion channels with the patch-clamp technique, which led ground-breaking discoveries in the 1990s.[27] Not only is the bandwidth remarkable, but also, the signal-to-noise level is at least a factor of 4 better at comparable time resolution than single-molecule FRET (Figure S10). The versatility and simplicity of plasmon rulers as tool to study single-protein conformational dynamics makes it possible to study slow and rare processes such as protein misfolding and denaturation or the complex conformational changes after or during the interaction with other proteins or small molecules. Comparing single-molecule traces will show the dynamical effects of the variability (non-ergodicity) of nominally identical protein species, caused, for example, by small differences in the secondary or tertiary structure or post-translational modifications. More fundamentally, long single-molecule traces open the window to investigate memory effects (non-Markovian behavior) and directionality (detailed balance).[23]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.8b03342.

Additional details, figures, and tables regarding the protein purification and labeling, nanoparticle synthesis, functionalization, flow cell preparation, plasmon ruler formation, the setup and setup performance, principal limits of the plasmon rulers study, decay rates, and comparisons with FRET (PDF)

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Author Contributions

W.Y. and M.G. contributed equally. C.S. and T.H. initiated and designed the research. The Hsp90 mutants were expressed and purified by M.G. and C.R.. The measurement setup and analysis software was developed by S.C. and W.Y. under the guidance of C.S., the dimer formation protocol was initially developed by J.P., then modified by L.T., both under the guidance of R.A-G. and C.S. The single molecule traces reported in this manuscript were measured by W.Y., L.T., S.C., M.G., R.A-G. The interpretation of the dimer traces and their statistical analysis was performed by W.Y., L.T., S.C., M.G., R.A-G, T.H. and C.S. The manuscript text was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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significant contributions to the setup control and data analysis software. Sebastian Schmachtel contributed to the dimer formation protocols. Arpad Jakab contributed significantly to the development of one of the setups. Jana Strugatchi and Johannes W. Sutter participated in some of the single-molecule measurements under the guidance of S.C. and W.Y.

REFERENCES

(1) Renaud, J.-P.; Chari, A.; Ciferri, C.; Liu, W.-T.; Rémigy, H.-W.; Stark, H.; Wiesmann, C. Cryo-EM in drug discovery: achievements, limitations and prospects. *Nat. Rev. Drug Discovery* **2018**, *17*, 471.

(2) Fernandez-Leiro, R.; Scheres, S. Unravelling biological macromolecules with cryo-electron microscopy. *Nature* **2016**, *537*, 339−346.

(3) Henzler-Wildman, K.; Kern, D. Dynamic personalities of proteins. *Nature* **2007**, *450*, 964−972.

(4) Neuman, K. C.; Nagy, A. Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy. *Nat. Methods* **2008**, *5*, 491−505.

(5) Roy, R.; Hohng, S.; Ha, T. A practical guide to single-molecule FRET. *Nat. Methods* **2008**, *5*, 507−516.

(6) Stigler, J.; Ziegler, F.; Gieseke, A.; Gebhardt, J. C. M.; Rief, M. The complex folding network of single calmodulin molecules. *Science* **2011**, *334*, 512−516.

(7) Sönnichsen, C.; Franzl, T.; Wilk, T.; von Plessen, G.; Feldmann, J.; Wilson, O.; Mulvaney, P. Drastic Reduction of Plasmon Damping in Gold Nanorods. *Phys. Rev. Lett.* **2002**, *88*, No. 077402-(1−4), DOI: 10.1103/PhysRevLett.88.077402.

(8) Sönnichsen, C.; Reinhard, B. M.; Liphardt, J.; Alivisatos, A. P. A Molecular Ruler Based on Plasmon Coupling of Single Gold and Silver Nanoparticles. *Nat. Biotechnol.* **2005**, *23*, 741−745.

(9) Reinhard, B. M.; Sheikholeslami, S.; Mastroianni, A.; Alivisatos, A. P.; Liphardt, J. Use of plasmon coupling to reveal the dynamics of DNA bending and cleavage by single EcoRV restriction enzymes. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 2667−2672.

(10) Morimura, H.; Tanaka, S.-I.; Ishitobi, H.; Mikami, T.; Kamachi, Y.; Kondoh, H.; Inouye, Y. Nano-Analysis of DNA Conformation Changes Induced by Transcription Factor Complex Binding Using Plasmonic Nanodimers. *ACS Nano* **2013**, *7*, 10733−10740.

(11) Jun, Y.-W.; Sheikholeslami, S.; Hostetter, D. R.; Tajon, C.; Craik, C. S.; Alivisatos, A. P. Continuous imaging of plasmon rulers in live cells reveals early-stage caspase-3 activation at the single-molecule level. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 17735−17740.

(12) Chen, T.; Hong, Y.; Reinhard, B. M. Probing DNA Stiffness through Optical Fluctuation Analysis of Plasmon Rulers. *Nano Lett.* **2015**, *15*, 5349−5357.

(13) Lee, S. E.; Chen, Q.; Bhat, R.; Petkiewicz, S.; Smith, J. M.; Ferry, V. E.; Correia, A. L.; Alivisatos, A. P.; Bissell, M. J. Reversible Aptamer-Au Plasmon Rulers for Secreted Single Molecules. *Nano Lett.* **2015**, *15*, 4564−4570.

(14) Tüting, L.; Ye, W.; Settanni, G.; Schmid, F.; Wolf, B. A.; Ahijado-Guzmán, R.; Sönnichsen, C. Potassium Triggers a Reversible Specific Stiffness Transition of Polyethylene Glycol. *J. Phys. Chem. C* **2017**, *121*, 22396−22402.

(15) Liu, N.; Hentschel, M.; Weiss, T.; Alivisatos, A.; Giessen, H. Three-Dimensional Plasmon Rulers. *Science* **2011**, *332*, 1407−1410.

(16) Mickler, M.; Hessling, M.; Ratzke, C.; Buchner, J.; Hugel, T. The large conformational changes of Hsp90 are only weakly coupled to ATP hydrolysis. *Nat. Struct. Mol. Biol.* **2009**, *16*, 281−286.

(17) Shiau, A. K.; Harris, S. F.; Southworth, D. R.; Agard, D. A. Structural analysis of E. coli Hsp90 reveals dramatic nucleotide-dependent conformational rearrangements. *Cell* **2006**, *127*, 329−340.

(18) Pearl, L. H.; Prodromou, C. Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annu. Rev. Biochem.* **2006**, *75*, 271−294.

(19) Ratzke, C.; Mickler, M.; Hellenkamp, B.; Buchner, J.; Hugel, T. Dynamics of heat shock protein 90 C-terminal dimerization is an important part of its conformational cycle. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 16101−16106.

(20) Hellenkamp, B.; Wortmann, P.; Kandzia, F.; Zacharias, M.; Hugel, T. Multidomain structure and correlated dynamics determined by self-consistent FRET networks. *Nat. Methods* **2017**, *14*, 174−180.

(21) Ali, M. M.; Roe, S. M.; Vaughan, C. K.; Meyer, P.; Panaretou, B.; Piper, P. W.; Prodromou, C.; Pearl, L. H. Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. *Nature* **2006**, *440*, 1013−1017.

(22) Schmid, S.; Götz, M.; Hugel, T. Single-Molecule Analysis beyond Dwell Times: Demonstration and Assessment in and out of Equilibrium. *Biophys. J.* **2016**, *111*, 1375−1384.

(23) Hu, X.; Hong, L.; Smith, M. D.; Neusius, T.; Cheng, X.; et al. The dynamics of single protein molecules is non-equilibrium and self-similar over thirteen decades in time. *Nat. Phys.* **2016**, *12*, 171−174.

(24) Wennmalm, S.; Edman, L.; Rigler, R. Non-ergodic behaviour in conformational transitions of single DNA molecules. *Chem. Phys.* **1999**, *247*, 61−67.

(25) Jahn, M.; Buchner, J.; Hugel, T.; Rief, M. Folding and assembly of the large molecular machine Hsp90 studied in single-molecule experiments. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 1232−1237.

(26) Borgia, M. B.; Borgia, A.; Best, R. B.; Steward, A.; Nettels, D.; Wunderlich, B.; Schuler, B.; Clarke, J. Single-molecule fluorescence reveals sequence-specific misfolding in multidomain proteins. *Nature* **2011**, *474*, 662−665.

(27) Verkhratsky, A.; Parpura, V. History of electrophysiology and the patch clamp. *Methods Mol. Biol.* **2014**, *1183*, 1−19.

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Nano Lett. 2018, 18, 6633−6637