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# Molecular Hyperthermia: Spatiotemporal Protein Unfolding and Inactivation by Nanosecond Plasmonic Heating

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*Spatiotemporal control of protein structure and activity in biological systems has important and broad implications in biomedical sciences as evidenced by recent advances in optogenetic approaches. Here, this study demonstrates that nanosecond pulsed laser heating of gold nanoparticles (GNP) leads to an ultrahigh and ultrashort temperature increase, coined as "molecular hyperthermia", which causes selective unfolding and inactivation of proteins adjacent to the GNP. Protein inactivation is highly dependent on both laser pulse energy and GNP size, and has a well-defined impact zone in the nanometer scale. It is anticipated that the fine control over protein structure and function enabled by this discovery will be highly enabling within a number of arenas, from probing the biophysics of protein folding/unfolding to the nanoscopic manipulation of biological systems via an optical trigger, to developing novel therapeutics for disease treatment without genetic modification.*

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Proteins are the functional machinery of biological systems, and the development of techniques to change a protein's conformation and control its function with both high spatial and temporal resolutions is a crucial pursuit in the development of next-generation medicine. This capability is being rigorously pursued as it offers significant and broad implications for uncovering a protein's folding and unfolding mechanisms, elucidating a protein's biological function in situ,[1–3] noninvasively manipulating biological activity,[4] and developing novel therapeutics for disease treatment. This is partially evidenced by the development and application of optogenetic approaches that have utilized light-responsive activity-modulating proteins in the last decade.[4,5] While the genetic modification represents a challenge for clinical translations, such novel approaches to spatiotemporally modify protein conformation and function promise to lead to paradigm shifting advances.

Nanoparticles offer a direct interface with proteins, and there have been significant advances in using nanoparticles for the long-term labeling and tracking of proteins in living cells owing to their excellent photostability.[6–8] While nanoparticle labeling has led to new understanding of protein localization and dynamics in live cells, the ability to further manipulate protein structure and activity with a high spatiotemporal resolution represent a significant advancement

  

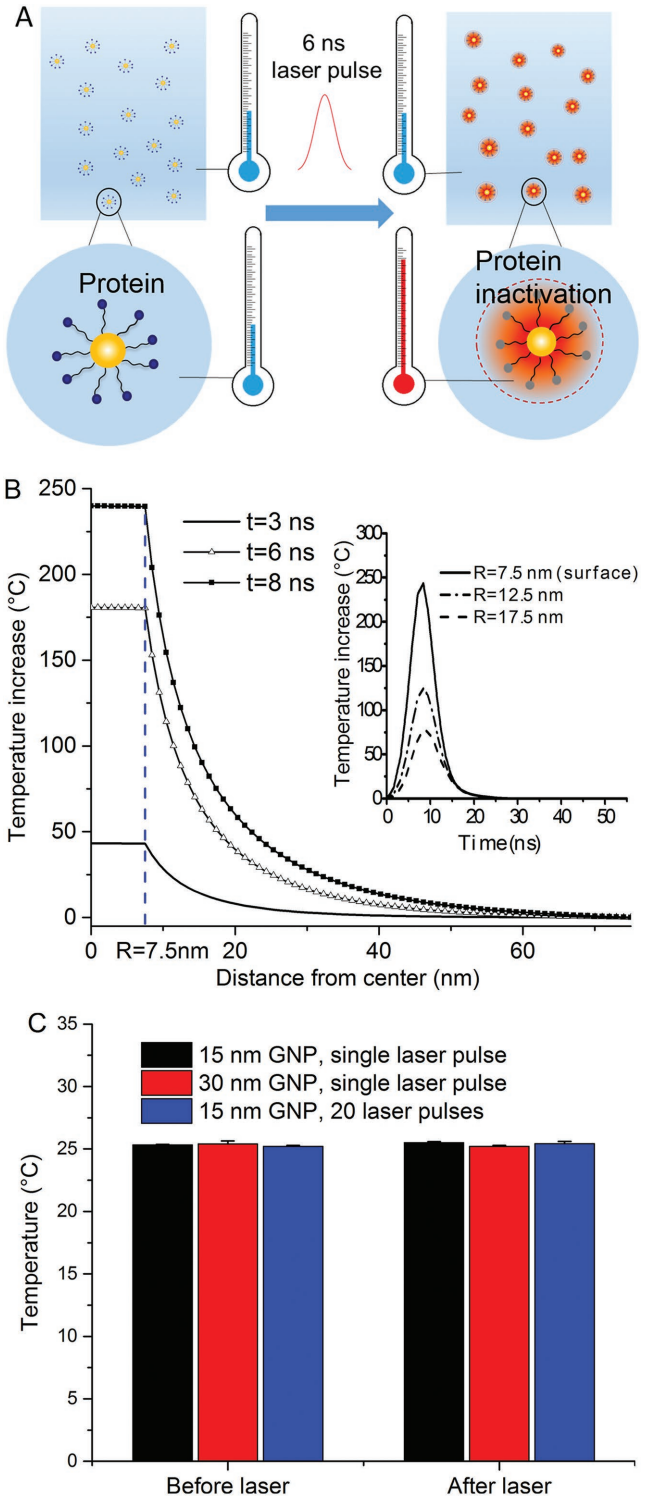


**Figure 1.** Thermal analysis of the plasmonic GNP heating with nanosecond laser pulses. A) The nanosecond laser pulse selectively heats up GNPs without increasing the solution temperature. B) Spatial and temporal temperature profile (from room temperature) of 15 nm gold nanoparticle in response to a 6 ns laser pulse with a Gaussian profile (87 mJ $cm^{-2}$). The heating is confined tens of nanometers within the GNP surface and lasts less than 25 ns (insert). The experimental conditions do not cause vapor bubble formation. C) The nanosecond heating does not change the bulk temperature of the GNP solution. The temperature was measured before and after laser pulse irradiation for 15 and 30 nm GNP solutions. The laser energy densities used here are consistent with the highest energy used in the protein inactivation experiment. Briefly, for single pulse, the energy densities of laser are 603 ± 5 mJ $cm^{-2}$ (15 nm GNP) and 611 ± 6 mJ $cm^{-2}$ (30 nm GNP). For 20 laser pulses, energy of 248 ± 4 mJ $cm^{-2}$ per pulse was used. There is no measurable bulk temperature change after laser irradiation even at the highest laser energy level.

with a wide range of new possibilities and applications. Continuous wave laser heating of plasmonic nanoparticles is known to induce a macroscopic temperature change and has been widely studied for photothermal hyperthermia therapy.[9] Recently, Thompson et al. demonstrated that gold nanorod heating under continuous wave laser leads to enzyme inactivation[10] and attributed this effect to the nanoscale heating effect in addition to the macroscopic heating. The laser heating increases the medium temperature by 10–20 °C; however, such a temperature change is insufficient to inactivate enzymes by holding the enzymes at equivalent temperatures without nanoparticles. While promising, the large temperature change may lead to changes in other cellular proteins at hyperthermic temperatures.

Pulsed laser heating of plasmonic gold nanoparticle (GNP) is known to create a highly localized heating.[11–15] Here, we utilize GNP and pulsed laser heating to generate a highly localized ultrahigh temperature in a few nanoseconds to cause ultrafast protein unfolding and inactivation, coined as "molecular hyperthermia". To test the idea, we conjugated an enzyme protein, α-chymotrypsin (Cht), to GNP through either the covalent bond or electrostatic absorption. By measuring and comparing the enzyme activity before and after the nanosecond laser irradiation, we determined the fraction of protein inactivated by the nanosecond plasmonic heating. The heating is localized within a few nanometers around the nanoparticle without changing the bulk medium temperature. We then systemically investigated the effects of GNP size, distance between GNP and protein, and laser pulse number on the protein inactivation, and found a well-defined protein inactivation impact zone around the GNP in the nanometer scale as a function of laser pulse energy and GNP size. We further examined the fate of GNP and GNP–protein conjugate after laser treatment and uncovered the GNP aggregation due to protein unfolding at high laser intensities. Both the high spatial (nanometers) and temporal (nanoseconds) capabilities offered by this new technique will find broad applications in the biophysical study of protein folding/unfolding, inactivation of protein activity in situ to elucidate protein function, remote manipulation of biological activity, and development of novel methods to treat diseases.

First, we analyzed the temperature response of GNPs and the surrounding medium in response to nanosecond laser pulses. As shown in **Figure 1**, the GNP sample was irradiated by a 532 nm nanosecond laser. When measuring the bulk solution temperature with a thermal couple, no temperature change in the medium was observed from the laser treatment (Figure 1C), indicating that the thermal response is limited to the nanoscale. We then analyzed the heating of an individual GNP by using GNP optical absorption properties

and solving the heat conduction equation.[16,17] Figure 1B shows the temperature profile of a single nanoparticle after a single 6 ns laser pulse exposure. The temperature of GNPs rises rapidly due to plasmonic absorption. The temperature with GNP is uniform due to the small size and high thermal conductivity (thus very small Biot number). The heat diffuses from GNP to the surrounding water and causes a significant temperature increase and steep temperature gradient within several nanometers around GNP. Importantly, the temperature rise is limited to the nanoparticle and its immediate surrounding medium, and is insufficient to heat up the entire solution (Figure 1C). Furthermore, the duration of the temperature change is less than 25 ns both on the nanoparticle surface and in the water around the particle. This molecular hyperthermia is fundamentally different from traditional hyperthermia or photothermal therapy, which requires macroscopic temperature changes that last seconds to minutes and lead to cell death within the entire tumor.[18] This nanoscale confinement can cause significant superheating to the water adjacent to the nanoparticle, and previous studies have confirmed that the water can be superheated to 85% of its critical temperature, or up to 276 °C, without vaporization.[11,19,20] The laser energy density values used in this study are considered below the vaporization threshold based on previous measurements (400 mJ $cm^{-2}$ for 250 nm gold nanoshells and 10 ns laser pulse, and dramatically increased vaporization threshold for smaller GNP).[21] To further confirm the nanoscale GNP heating, we measured the photoacoustic signal (Figure S1, Supporting Information) corresponding to thermoelastic expansion of the GNP and water,[22] which is a result of the nanoscale heating. Furthermore, gold nanocrystals have vortexes and edges (Figure S5, Supporting Information) and lead to higher heat generation[23] and altered temperature distribution of water around the GNP. Further investigation is required to elucidate the effect of GNP shape on the nanosecond thermal responses and protein inactivation.

Next, we demonstrated experimentally that proteins adjacent to GNP can be efficiently and selectively unfolded and inactivated by molecular hyperthermia. GNP with a diameter of 15 nm was synthesized. First, GNP was mixed with egg white and treated by the nanosecond laser pulse. Compared with bulk heating (100 °C, 10 min) that causes egg white protein unfold and aggregate (i.e., opaque appearance), molecular hyperthermia does not change sample macroscopic properties (i.e., stays transparent, **Figure 2**A). This shows that the nanosecond laser pulse selectively causes nanoscale protein unfolding and inactivation close to GNP, without affecting most proteins that are not bound to GNP. To further investigate the selective protein inactivation, proteins were either absorbed directly to the GNP or conjugated with a polyethylene glycol (PEG, 1 kDa) spacer between the protein and GNP. Here, we used a protein enzyme, Cht, as its activity can be readily measured by a colorimetric assay. Approximately 15 Cht molecules are bound to one 15 nm GNP (Figure S4, Supporting Information). To check the Cht thermal stability, circular dichroism spectrum of Cht was performed (Figure S2, Supporting Information). The denaturation temperature for Cht $\alpha$ helix structure was found to be 59 °C under both bulk and slow heating. After applying a single 6 ns laser pulse, the activity of Cht–GNP conjugate showed a laser energy-dependent inactivation (Figure 2B). When Cht was not conjugated to the GNP or no GNP was present, the protein remained intact and catalytically active. The addition of the 1 kDa PEG spacer between GNP and Cht required an increase in the laser energy to achieve similar level of protein inactivation. Specifically, the 1 kDa PEG spacer increased the laser pulse energy required for 50% of protein inactivation from 82 to 127 mJ $cm^{-2}$. This is because the presence of PEG molecules increases the distance between Cht molecules and GNP. The PEG spacer length can be calculated using a previously reported method,[24] leading to an estimated PEG layer thickness in the range of 2.28 nm (mushroom structure) to 7.95 nm (fully expanded) as detailed in the Supporting information. Since the temperature decreases rapidly from the GNP surface (Figure 1B), the local temperature change that the protein experiences in the presence of a PEG spacer is much lower than the case without the PEG spacer. For instance, as shown in Figure 1B, the temperature increase within 6 ns at 5 nm from GNP surface (88 °C) is only about 48.9% of GNP surface temperature increase (180 °C).

We further studied different GNP sizes (5, 15, and 30 nm) and found that the larger 30 nm GNP requires less laser energy for Cht inactivation compared with smaller 5 and 15 nm (Figure 2C). The laser pulse energy required for 50% protein inactivation reduced from 127 mJ $cm^{-2}$ for 15 nm GNP to 47 mJ $cm^{-2}$ for 30 nm GNP. Interestingly for 5 nm GNP, the protein activity remained at 66% even at a very high laser pulse energy density 576 mJ $cm^{-2}$, while all protein activity was lost when conjugated to 15 and 30 nm GNP. This clearly demonstrates that the protein inactivation due to the nanoscale plasmonic heating is strongly size dependent, since large GNP absorbs more laser energy than smaller GNPs and thus leads to higher temperature changes. Next, we examined the use of multiple laser pulses (Figure 2D). The catalytic efficiency of Cht continued to decrease for up to ten laser pulses, beyond which no further loss in protein activity was observed. The final protein inactivation at ten laser pulses shows a strong laser pulse energy dependence. Finally, we demonstrated that protein inactivation is specific to the protein that is tagged or linked to the GNP in solution. By linking GNP to Cht but not alkaline phosphatase (ALP), the Cht activity was selectively reduced without significantly affecting the function of ALP. As shown in Figure 2E, one laser pulse decreased the Cht activity to 9%, but ALP activity still stayed high at 79%. The slight decrease in the ALP activity may be due to some passive absorption of ALP to GNP.

Furthermore, we analyzed the kinetics of molecular hyperthermia by nanosecond plasmonic heating-induced protein inactivation. Molecular dynamic simulations of protein unfolding at elevated temperatures (up to 550 K) have suggested that proteins can unfold as fast as within nanoseconds.[25,26] However, the experimental measurement at such short time scales has been challenging. Earlier, *T*-jump experiments measured protein unfolding kinetics within milliseconds,[27] and recently, a laser gold film heating

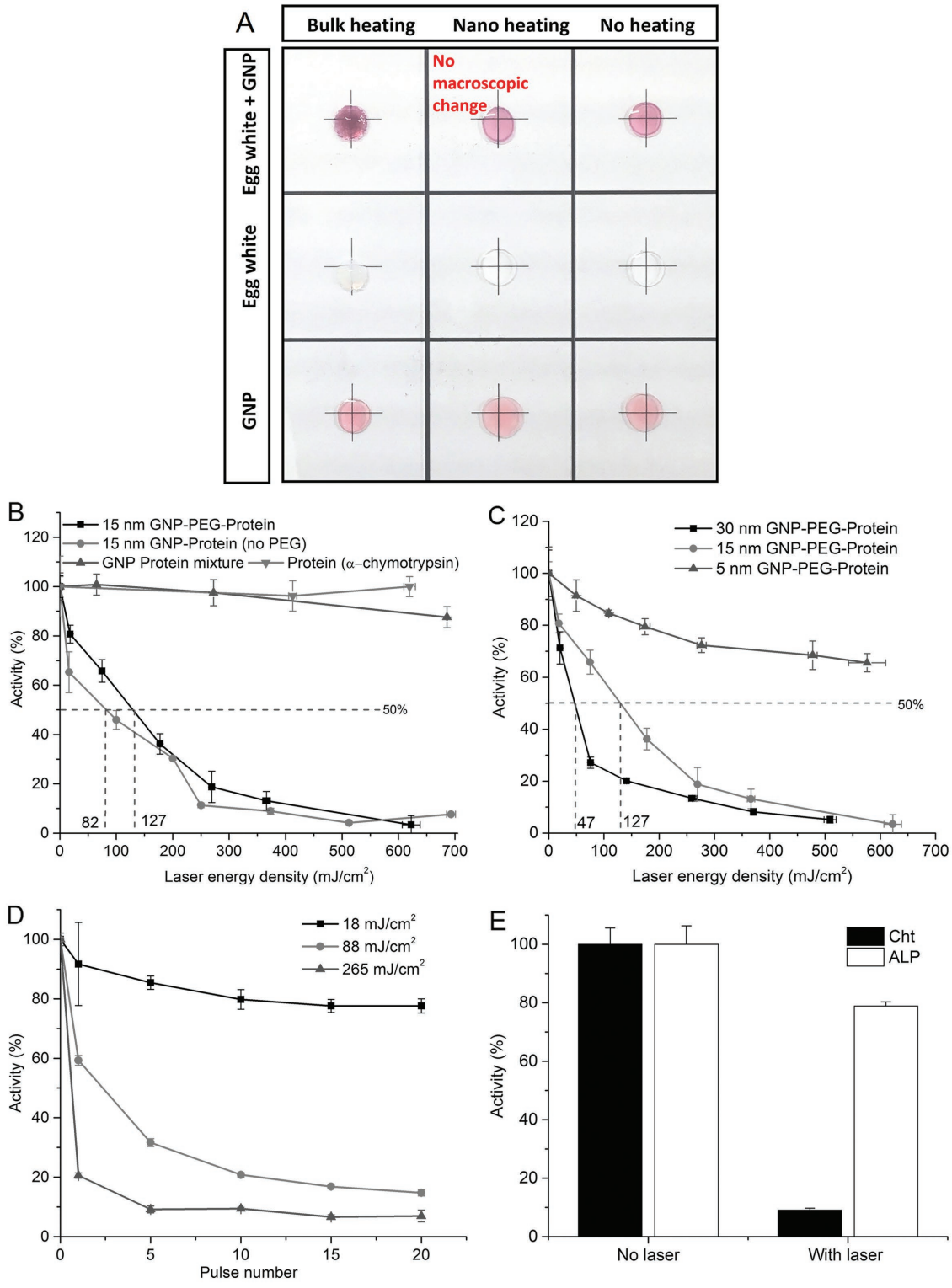


**Figure 2.** Experimental characterization of the nanosecond protein inactivation. A) The nanosecond protein inactivation is specific to proteins around the GNP surface. Inactivation or denaturation of egg white makes itself opaque. First column shows bulk heating of the egg white–GNP mixture, egg white only, and GNP only, while the second and third columns show the nanoscale heating and no treatment for the same samples, respectively. The bulk heating changes the egg white and egg white–GNP mixture to an opaque appearance, compared with the transparent appearance of nanoscale heating treatment and no treatment. A background cross label is used for visualization. Laser pulse energy density is $114 \pm 4$ mJ cm$^{-2}$ for the nanoscale heating. B) Effect of the distance between GNP and protein on the protein inactivation. Legends: Protein refers to $\alpha$-chymotrypsin (Cht); GNP protein mixture refers to simply mixing Cht with the polyethylene glycol (PEG)-protected GNP without conjugation; 15 nm GNP-PEG-Protein refers to $\alpha$-chymotrypsin conjugated onto GNP via 1 kDa PEG spacer; 15 nm GNP-Protein refers to $\alpha$-chymotrypsin conjugated to GNP by electrostatic absorption. C) Effect of GNP size on the protein inactivation (diameter, 5, 15, and 30 nm). Protein is conjugated to GNP

study by Steel et al. confirms protein unfolding and inactivation within tens of nanoseconds.[28] Further comparison of the measured protein inactivation rate suggests that protein inaction follows an Arrhenius behavior, although the inactivation rate spans over 10 orders of magnitude ($k$, $10^{-3}$–$10^{8}$ $s^{-1}$) in a wide temperature range. As an approximation, the nanosecond protein inactivation around GNP was calculated using the Arrhenius kinetics (activation energy = 244.05 kJ $mol^{-1}$ and frequency factor = $9.75 \times 10^{38}$ $s^{-1}$).[27] The result suggests that the combination of the rapid temperature rise and the sharp temperature gradient creates a highly localized region of protein inactivation next to the nanoparticle surface, referred to as the impact zone. Here, we quantitatively define the impact zone ($z$) as the distance between the GNP surface to the position where the protein has 50% of its original activity after the nanosecond laser pulse treatment (**Figure 3**A):

$$z = R_{50\%} - R_{GNP} \quad (1)$$

By analyzing a range of GNP sizes (5–70 nm) and laser pulse energies, the impact zone ranges from 0–16 nm (Figure 3B). Larger nanoparticles require less laser intensity to inactivate proteins and also generate larger impact zones, due to the higher laser energy absorption and lower surface-to-volume ratio for heat dissipation. Very small 5 nm GNP gives a very small impact zone and thus does not inactivate proteins efficiently as evidenced by our measurement (Figure 2C). Also, increasing laser energy density for the same GNP size increases the impact zone due to the higher temperature which accelerates protein unfolding and inactivation, consistent with our experimental observations in Figure 2. Here, we note that the increase in laser energy is limited by the possibility of vaporization which has been shown to repel the protein from GNP surface with shorter picosecond laser pulse durations.[29] Further comparison of the theoretical analysis and experimental measurement shows that the laser energy for the protein inactivation is on the same order of magnitude (tens to hundreds of mJ $cm^{-2}$). Here, the location of the protein was taken as 1.9 nm based on a spherical protein shape approximation.[30] The length of the 1 kDa PEG spacer was estimated to be approximately 4.0 nm for 5 nm GNP, 3.9 nm for 15 nm GNP, and 5.8 nm for 30 nm GNP based on hydrodynamic measurements by dynamic light scattering (DLS) (Figure S3, Supporting Information). The slight difference in the PEG length estimation may be attributed to the different footprints of PEG on the two GNP sizes.[31]

Lastly, we examined the fate of GNP and GNP–protein conjugate after laser treatment. By comparing TEM images (Figure S4, Supporting Information) before and after the laser treatment, no significant GNP structure change was observed at moderate laser intensities (137 mJ $cm^{-2}$) while

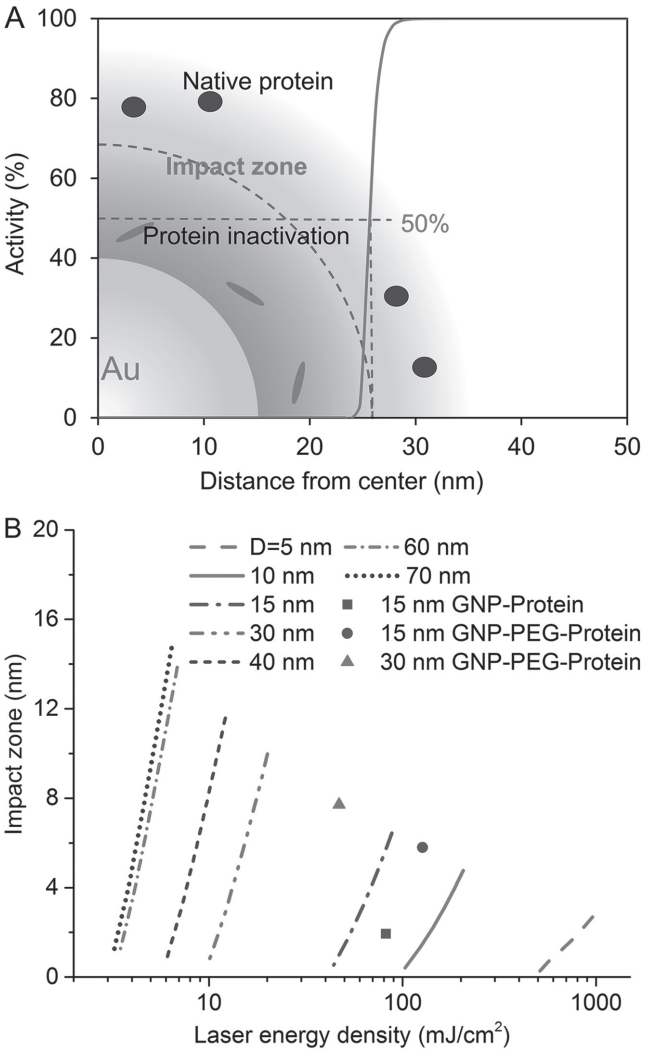


**Figure 3.** Impact zone for the nanosecond laser pulse-induced protein inactivation around GNPs. A) Schematic of the protein inactivation impact zone and analytical result of the predicted protein activity due to the nanoscale heating. B) Effect of laser energy and GNP size on the impact zone.

GNP fragmentation was observed at high laser intensity (≈600 mJ $cm^{-2}$). This is consistent with previous reports on the laser fragmentation of plasmonic nanoparticles with high laser intensities.[32] Interestingly, when comparing PEG and Cht-conjugated GNP, the PEG coating stabilizes the GNP while the Cht–GNP conjugate shows significant aggregation after high energy laser pulse treatment, as seen in the DLS (**Figure 4**), agarose gel electrophoresis (Figure S6, Supporting Information), and UV–vis measurements (Figure S7, Supporting Information). This is possibly a result of protein unfolding, which exposes the hydrophobic interior and leads to the aggregation of the Cht–GNP conjugate, similar to the protein aggregation after complete unfolding[33,34] (Figure S8, Supporting Information).

surface via a 1 kDa PEG spacer. D) Effect of laser pulse number on the protein inactivation. E) Specificity of the nanosecond protein inactivation. ALP refers to alkaline phosphatase. Cht is conjugated to GNP surface while ALP is not conjugated to GNP surface. Laser energy density is 510 ± 25 mJ $cm^{-2}$, and GNP size is 15 nm.

  

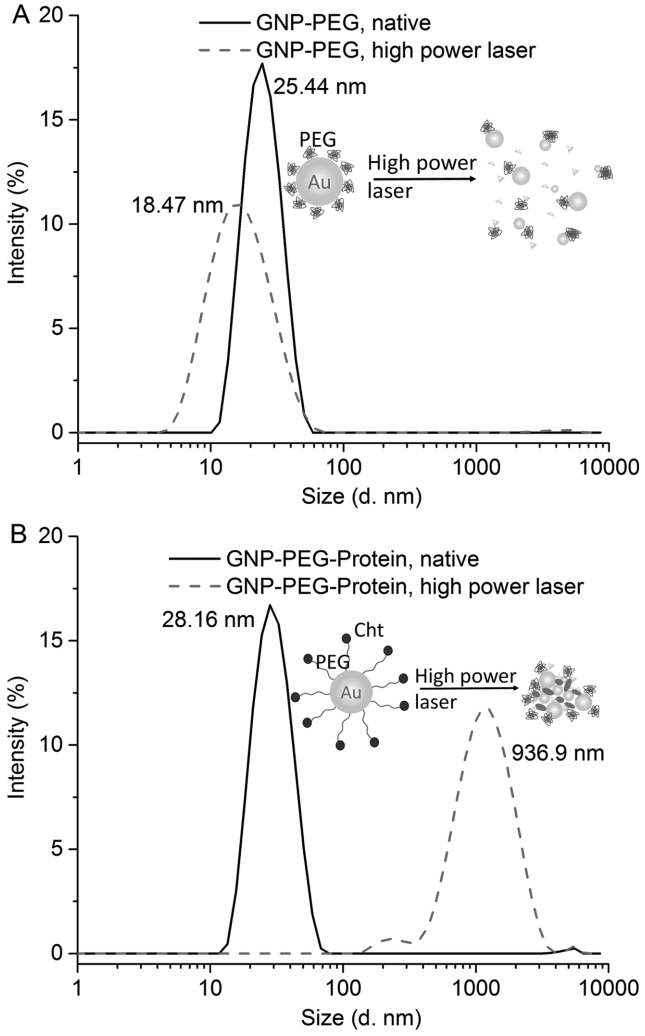


**Figure 4.** Protein unfolding leads to aggregation of protein–GNP conjugate at high laser intensities. A) PEG-coated GNP is stable under all laser intensities attempted (up to ≈609 mJ cm$^{-2}$). B) High laser intensity (609 mJ cm$^{-2}$) leads to protein–GNP conjugate aggregation while moderate laser intensity (137 mJ cm$^{-2}$) does not cause aggregation.

In conclusion, molecular hyperthermia by nanosecond plasmonic GNP heating selectively unfolds and inactivates targeted proteins and offers a novel platform to remotely manipulate protein structure and activity without genetic modification. The effects of GNP size, laser energy, and pulse number on the protein inactivation process were systematically studied. Our findings not only quantified the protein inactivation kinetics but also revealed the aggregation behavior of protein–GNP conjugates at high laser intensities and thus significant protein unfolding. Further work is warranted to investigate the ultrafast kinetics of the protein unfolding by time-resolved X-ray scattering[35–37] and detailed structural change using hydrogen/deuterium exchange and mass spectrometry.[38–40] In particular, further work is needed to understand the mechanism and kinetics of protein unfolding at these extremely high temperatures and very short time scales (nanoseconds). Furthermore, the selective protein inactivation combined with nanomaterial labeling may prove useful for basic protein functional research,[41–43] remote control of biological activity and behavior, and in therapeutic applications.

## Supporting Information

*Supporting Information is available from the Wiley Online Library or from the author.*

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## Conflict of Interest

*The authors declare no conflict of interest.*

[1] J. Kubelka, *Photochem. Photobiol. Sci.* **2009**, *8*, 499.
[2] V. Daggett, *Chem. Rev.* **2006**, *106*, 1898.
[3] K. M. Marks, P. D. Braun, G. P. Nolan, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9982.
[4] L. Fenno, O. Yizhar, K. Deisseroth, *Annu. Rev. Neurosci.* **2011**, *34*, 389.
[5] A. Berndt, S. Y. Lee, J. Wietek, C. Ramakrishnan, E. E. Steinberg, A. J. Rashid, H. Kim, S. Park, A. Santoro, P. W. Frankland, S. M. Iyer, S. Pak, S. Ährlund-Richter, S. L. Delp, R. C. Malenka, S. A. Josselyn, M. Carlén, P. Hegemann, K. Deisseroth, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 822.
[6] C. Leduc, S. Si, J. Gautier, M. Soto-Ribeiro, B. Wehrle-Haller, A. Gautreau, G. Giannone, L. Cognet, B. Lounis, *Nano Lett.* **2013**, *13*, 1489.
[7] L. Cognet, C. Tardin, D. Boyer, D. Choquet, P. Tamarat, B. Lounis, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 11350.
[8] D. J. Nieves, Y. Li, D. G. Fernig, R. Lévy, *R. Soc. Open Sci.* **2015**, *2*, 140454.
[9] X. Huang, P. K. Jain, I. H. El-Sayed, M. A. El-Sayed, *Lasers Med. Sci.* **2008**, *23*, 217.
[10] S. A. Thompson, S. Paterson, M. M. M. Azab, A. W. Wark, R. de la Rica, *Small* **2017**, *13*, 1603195.
[11] Z. Qin, J. C. Bischof, *Chem. Soc. Rev.* **2012**, *41*, 1191.
[12] P. Berto, M. S. A. Mohamed, H. Rigneault, G. Baffou, *Phys. Rev. B: Condens. Matter Mater. Phys.* **2014**, *90*, 1.
[13] A. O. Govorov, W. Zhang, T. Skeini, H. Richardson, J. Lee, N. A. Kotov, *Nanoscale Res. Lett.* **2006**, *1*, 84.
[14] G. Hüttmann, B. Radt, J. Serbin, R. Birngruber, *Proc. SPIE* **2003**, *5142*, 88.
[15] C. M. Pitsillides, E. K. Joe, X. Wei, R. R. Anderson, C. P. Lin, *Biophys. J.* **2003**, *84*, 4023.
[16] H. Goldenberg, C. J. Tranter, *Br. J. Appl. Phys.* **1952**, *296*, 296.
[17] Z. Qin, Y. Wang, J. Randrianalisoa, V. Raeesi, W. C. W. Chan, W. Lipiński, J. C. Bischof, *Sci. Rep.* **2016**, *6*, 29836.

  

[18] L. R. Hirsch, R. J. Stafford, J. A. Bankson, S. R. Sershen, B. Rivera, R. E. Price, J. D. Hazle, N. J. Halas, J. L. West, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13549.
[19] A. Siems, S. A. L. Weber, J. Boneberg, A. Plech, *New J. Phys.* **2011**, *13*, 43018.
[20] M. T. Carlson, A. J. Green, H. H. Richardson, *Nano Lett.* **2012**, *12*, 1534.
[21] E. Lukianova-Hleb, Y. Hu, L. Latterini, L. Tarpani, S. Lee, R. A. Drezek, J. H. Hafner, D. O. Lapotko, *ACS Nano* **2010**, *4*, 2109.
[22] G. A. Pang, J. Laufer, R. Niessner, C. Haisch, *J. Phys. Chem. C* **2016**, *120*, 27646.
[23] R. Rodríguez-Oliveros, J. A. Sánchez-Gil, *Opt. Express* **2012**, *20*, 621.
[24] J. L. Perry, K. G. Reuter, M. P. Kai, K. P. Herlihy, W. Stephen, J. C. Luft, M. Napier, J. E. Bear, M. Joseph, *Nano Lett.* **2014**, *12*, 5304.
[25] C. Yan, V. Pattani, J. W. Tunnell, P. Ren, *J. Mol. Graphics Modell.* **2010**, *29*, 2.
[26] H. S. Chung, M. Khalil, A. W. Smith, Z. Ganim, A. Tokmakoff, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 612.
[27] G. Hüttmann, R. Birngruber, *IEEE J. Sel. Top. Quantum Electron.* **1999**, *5*, 954.
[28] B. C. Steel, D. R. McKenzie, M. M. M. Bilek, N. J. Nosworthy, C. G. dos Remedios, *Biophys. J.* **2006**, *91*, L66.
[29] S. Ibrahimkutty, J. Kim, M. Cammarata, F. Ewald, J. Choi, H. Ihee, A. Plech, *ACS Nano* **2011**, *5*, 3788.
[30] H. P. Erickson, *Biol. Proced. Online* **2009**, *11*, 32.
[31] K. Rahme, L. Chen, R. G. Hobbs, M. A. Morris, C. O'Driscoll, J. D. Holmes, *RSC Adv.* **2013**, *3*, 6085.
[32] F. Mafuné, J. Kohno, Y. Takeda, T. Kondow, *J. Phys. Chem. B* **2003**, *107*, 12589.
[33] D. Matulis, J. K. Kranz, F. R. Salemme, M. J. Todd, *Biochemistry* **2005**, *44*, 5259.
[34] S. Dominguez-Medina, L. Kisley, L. J. Tauzin, A. Hoggard, B. Shuang, A. S. D. S. Indrasekara, S. Chen, L.-Y. Wang, P. J. Derry, A. Liopo, E. R. Zubarev, C. F. Landes, S. Link, *ACS Nano* **2016**, *10*, 2103.
[35] H. D. T. Mertens, D. I. Svergun, *J. Struct. Biol.* **2010**, *172*, 128.
[36] M. Cammarata, M. Levantino, F. Schotte, P. A. Anfinrud, F. Ewald, J. Choi, A. Cupane, M. Wulff, H. Ihee, *Nat. Methods* **2008**, *5*, 881.
[37] D. Arnlund, L. C. Johansson, C. Wickstrand, A. Barty, G. J. Williams, E. Malmerberg, J. Davidsson, D. Milathianaki, D. P. DePonte, R. L. Shoeman, D. Wang, D. James, G. Katona, S. Westenhoff, T. A. White, A. Aquila, S. Bari, P. Berntsen, M. Bogan, T. B. van Driel, R. B. Doak, K. S. Kjær, M. Frank, R. Fromme, I. Grotjohann, R. Henning, M. S. Hunter, R. A. Kirian, I. Kosheleva, C. Kupitz, M. Liang, A. V. Martin, M. M. Nielsen, M. Messerschmidt, M. M. Seibert, J. Sjöhamn, F. Stellato, U. Weierstall, N. A. Zatsepin, J. C. H. Spence, P. Fromme, I. Schlichting, S. Boutet, G. Groenhof, H. N. Chapman, R. Neutze, *Nat. Methods* **2014**, *11*, 923.
[38] A. J. Percy, M. Rey, K. M. Burns, D. C. Schriemer, *Anal. Chim. Acta* **2012**, *721*, 7.
[39] G. Wang, I. A. Kaltashov, *Anal. Chem.* **2014**, *86*, 7293.
[40] Z. Zhang, D. L. Smith, *Protein Sci.* **2008**, *2*, 522.
[41] M. S. Robinson, D. A. Sahlender, S. D. Foster, *Dev. Cell* **2010**, *18*, 324.
[42] J. Lin, S. Sann, K. Zhou, S. Nabavi, C. Proulx, R. Malinow, Y. Jin, R. Tsien, *Neuron* **2013**, *79*, 241.
[43] K. Jacobson, Z. Rajfur, E. Vitriol, K. Hahn, *Trends Cell Biol.* **2008**, *18*, 443.

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