Spatial and Temporal Confined Photothermolysis of Cancer Cells Mediated by Hollow Gold Nanospheres Targeted to Epidermal Growth Factor Receptors

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Supporting Information

ABSTRACT: To date, a few studies have investigated the potential use of a short-pulsed laser in selective tumor cell destruction or its mechanism of cell killing. Computer simulation of the spatial and temporal profiles of temperature elevation after pulsed laser irradiation on an infinitesimal point source estimated that the temperature reached its highest point at ∼35 ns after a single 15 ns laser pulse. Moreover, temperature elevation was confined to a radius of sub-micrometer and returned to baseline within 100 ns. To investigate the effect of 15 ns laser pulses on A431 tumor cells, we conjugated hollow gold nanospheres (HAuNSs) to an antibody (C22S) directed at the epithelial growth factor receptor. The resulting nanoparticles, C22S-HAuNSs, bound to the cell membrane, internalized, and distributed throughout the cytoplasm, with some nanoparticles transported to the vicinity of the nuclear membrane. On using an optical microscope mounted to a tunable pulsed Ti:sapphire laser, rapid and extensive damage of live cancer cells was observed, whereas irradiation of A431 cells pretreated with nontargeted HAuNSs with a pulsed laser or pretreated with C22S-HAuNSs with a continuous-wave laser-induced minimal cellular damage. Furthermore, after a single 15 ns laser pulse, C22S-HAuNS-treated A431 cells cocultured with 3T3 fibroblasts showed signs of selective destruction. Thus, compared with a continuous-wave laser, shots of a short-pulsed laser were the most damaging to tumor cells that bound HAuNSs and generated the least heat to the surrounding environment. This mode of action by a short-pulsed laser on cancer cells (i.e., confined photothermolysis) may have potential applications in selective tumor cell destruction.

1. INTRODUCTION

Selective photothermolysis using short laser pulses was first proposed by Anderson and Parrish in 1983,1 and the theory of selective thermal damage was extended in 2001 by Altshuler et al.2 Since then, the use of pulsed lasers has been successfully translated into the clinic in dermatology and laser treatment of skin lesions has become a mainstay in the cosmetic industry.3 Intrinsic biological chromophores, such as melanin and hemoglobin, absorb laser light and generate photothermolysis to induce pigment bleaching and cell damage. Photothermolysis is controlled in a spatiotemporal fashion to obtain optimal effect on a targeted tissue with minimal effect on the surrounding tissue.

Metallic nanoparticles such as gold nanorods and gold nanoshells are a new type of optical absorbers that display strong optical absorption owing to surface plasmon resonance. The peak absorption of these metallic nanoparticles can be tuned to the near-infrared (NIR) region. It has been reported that a short-pulsed laser can induce the formation of nanobubbles around nanoparticles.4−7 Cells with metallic plasmonic nanoparticles that are irradiated with a pulsed laser are believed to be destroyed by the expansion and collapse of nanobubbles, and such a phenomenon is referred to as a photomechanical effect.8,9

However, the mechanism(s) of cell killing and the impact of photon−nanoparticle interaction on cancer cells remain to be clarified. The current observations of nanoscale phenomena on the basis of optical scattering images and transient acoustic signal recordings are indirect and inconclusive. For example, the detected acoustic pressure is believed to be a contributing factor of the nanobubbles generated. However, the formation of nanobubbles may also result from nanoparticle expansion under the pulsed laser or vaporization of water with a rapid temperature rise in highly confined spatial and temporal scales. The recorded scattering image may also be attributed to nanoparticle expansion and Brownian movement upon irradiation with a pulsed laser. In addition to laser-induced nanobubbles and their subsequently generated photomechanical force, photothermal energy may eventually diffuse from nanoparticles to their adjacent environment to mediate cellular

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The thermalization process is a few hundred femtoseconds.\textsuperscript{16,19} To electron-to-electron scattering. The temporal scale of this process is said to be on the order of nano-seconds.\textsuperscript{10} The absorption of light energy and reaching internal thermal equilibrium due to electron-hole pair formation and electron-electron scattering is described as reaching local thermalization, which takes a few picoseconds.\textsuperscript{1} The characteristic time for relaxation varies from 10 ps for the smallest particles examined to almost 400 ps for nanoparticles of 50 nm in diameter.\textsuperscript{25} The optical energy to thermal energy is eventually dissipated as heat into the surrounding medium. The heat capacity of blood ($C_p = 3617 \text{ J/(kg K)}$), blood density ($\rho = 1050 \text{ kg/m}^3$), and thermal conductivity of blood ($K = 0.52 \text{ W/(m K)}$). The calculated thermal diffusivity was $1.369 \times 10^{-7} \text{ m}^2/s$, and the calculated thermal confinement size was $5.23 \times 10^{-8} \text{ m}$ or 52 nm in diameter. Beyond the radius of 0.1 \text{μm}, there was no significant temperature increase.

2. RESULTS AND DISCUSSION

The temperature change around a nanoparticle in a cellular environment as a function of both position $r$ spatially and time $t$ temporally can be obtained by solving a thermal convection equation. For simplicity, we assume that the hollow gold nanosphere (HAuNS) nanoparticle is a perfect spherical point and that heat flux has spherical symmetry, thereby simplifying a three-dimensional problem into a one-dimensional problem. The energy distribution of a single-pulsed laser was assumed to be Gaussian. The pulsed volumetric heating of a single pulse was thus expressed as

$$P(t) = P_0 \exp \left(-\frac{(t - t_0)^2}{\tau^2}\right)$$

where $\tau$ is the pulse duration of the full width at half-maximum. The laser has peak power $P_0$ at time $t_0$. This equation can be solved once boundary and initial conditions are defined. Using numerical methods, we obtained temperature elevation as a function of both position $r$ and time $t$. Figure 1 shows the simulated temperature profiles of an infinitesimal point source absorbs and transports laser energy to the surrounding medium, causing the temperature increase. With the use of laser pulses with a 15 ns pulsewidth, our simulation indicated that most laser energy is converted into thermal energy. The temperature started to increase for a few picoseconds after the laser shot and increased in both amplitude and width to peak at 35 ns. Then, the temperature diffused with a decreased amplitude but an increased width. The spatial range of the photothermal effect was estimated according to thermal conduction and diffusion equations. The temperature increase was confined in the characteristic spatial dimension of 52 nm. Beyond a radius of 100 nm (0.1 μm), there was no significant temperature increase. Our results support the concept that the interaction of nanosecond laser pulses with plasmonic metallic nanoparticles primarily mediates the conversion of optical energy into both thermal and mechanical (acoustic) energy by a nanoparticle. The conversion efficiencies to each energy form are determined by laser pulsewidth and, perhaps more importantly, the rising and falling slope of the laser pulse. Ostrovskaya\textsuperscript{16,27} studied the effects of laser-to-acoustic energy conversion on the interaction of pulsed laser radiation with a liquid medium and presented a theoretical treatment for absolute values of the efficiency for various laser properties.

![Figure 1. Simulation of temperature profiles and thermal diffusion profiles as a function of distance from a point absorber upon irradiation with a 15 ns laser pulse. An infinitesimal point source absorbs and transports laser energy to the surrounding medium to cause temperature elevation. (A) After a single laser pulse, the temperature elevation peaked at ~35 ns. Afterward, the temperature gradually decreased and returned to the baseline level over a course of ~100 ns. (B) Owing to heat diffusion, the temperature initially increased in both amplitude and width (solid lines). After the temperature elevation peaked at 35 ns, the temperature decreased in amplitude but increased in width. The simulation conditions we used were as follows: heat capacity of blood ($C_p = 3617 \text{ J/(kg K)}$), blood density ($\rho = 1050 \text{ kg/m}^3$), and thermal conductivity of blood ($K = 0.52 \text{ W/(m K)}$). The calculated thermal diffusivity was $1.369 \times 10^{-7} \text{ m}^2/s$, and the calculated thermal confinement size was $5.23 \times 10^{-8} \text{ m}$ or 52 nm in diameter. Beyond the radius of 0.1 \text{μm}, there was no significant temperature increase.](image-url)
Figure 2. Cellular distribution of C225-HAuNSs in A431 cells. (A, B) Representative transmission electron micrographs of A431 cells showing intracellular distribution of C225-HAuNSs. (A-i) Overview of cellular distribution of C225-HAuNSs. C225-HAuNSs were found on the cell membrane (A-ii), in the cytoplasm adjacent to the nucleus (A-iii), and in the cytoplasmic space enclaved by the nuclear membrane (A-iv). Red arrows: C225-HAuNS nanoparticles. (B) C225-HAuNSs were found in the close proximity to the nuclear membrane. Images shown in B-i–iv were taken at increasing magnification. (C) Quantification of intracellular distribution of C225-HAuNS in A431 cells. The data are expressed as mean ± standard deviation calculated from three cells.

Figure 3. Representative microphotographs of A431 cells treated with C225-HAuNs followed by one and two shots of 15 ns 780 nm laser pulses. The second laser pulse was instituted at 25 s after the first laser pulse. Under white light illumination, the greenish HAuNS clusters turned brownish. Microbubbles (red arrows) formed and subsequently collapsed. After the second laser pulse, additional microbubbles formed. During the course of first and second laser pulses, blebs were shed off from the cell membrane. Blue arrowheads: blebs shedding from laser-irradiated cells.
(e.g., short pulse and long pulse) with boundary conditions and nonlinearity. The photomechanical conversion efficiency was found to be on the scale of $10^{-7}$. In the presence of metallic nanoparticles, previous attempts describing both mechanical and thermal effects of tissue interaction with nanosecond laser pulses are likely incorrect because the transfer and conversion of all of the energy are expected to occur on the spatial scale of nanometers and on the temporal scale of nanoseconds.

Although a low photomechanical conversion efficiency is anticipated in a photon–nanoparticle interaction, metallic nanoparticles have been widely reported as contrast agents for photoacoustic imaging (PAI). PAI is a fast-developing biomedical imaging modality based on the detection of a photomechanical wave, also referred to as a photoacoustic wave, induced by the optical absorption of nanosecond laser pulses. Selective uptake of nanoparticles in cancer cells through receptor-mediated endocytosis enhances the optical absorption of cancer cells, resulting in strong photoacoustic signals with high sensitivity and specificity for cancer cell detection. Nevertheless, according to our simulation, most optical energy of laser pulses having a pulsewidth in the magnitude of nanoseconds is transformed into thermal energy by the nanoparticles. Therefore, to achieve a higher photomechanical conversion efficiency for PAI in the presence of metallic nanoparticles, femtosecond laser pulses should be considered to generate greater photomechanical effects.

To understand the cellular destruction process after irradiation with nanosecond laser pulses, we used HAuNS as the NIR light absorber and conjugated the anti-EGFR antibody (C225) to HAuNS to mediate targeted binding and cellular internalization of HAuNSs. Transmission electron micrographs of A431 cells treated with C225-HAuNSs revealed the intracellular distribution of HAuNSs in the cells (Figure 2). HAuNS nanoparticles were identified as high-density spherical particles of $\sim 40-50$ nm in diameter at high magnification ($100,000 \times$) and displayed a typical hollow shell structure with 40–50 nm diameter and $\sim 7$ nm shell thickness. Each A431 cell contained on an average $1600 \pm 398$ C225-HAuNS nanoparticles. More than half of these nanoparticles ($56 \pm 14\%$) were bound to the cell membrane (Figure 2A,C), whereas the remaining nanoparticles were distributed throughout the cytoplasm (Figure 2A,B). In the cytoplasm, C225-HAuNSs often formed clusters of nanoparticles enclosed in intracellular vesicles forming gold rings with a diameter of $\sim 0.5 \mu m$ (Figure 2A-iv,B-iv). Presumably, these C225-HAuNS nanoparticles were internalized through receptor-mediated endocytosis and localized in endolysosomes. Thus, C225-HAuNS nanoparticles were effectively taken up by A431 cells.

Interestingly, a fraction of nanoparticle clusters ($4.1 \pm 0.7\%$) in these cytoplasmic vehicles was transported to areas in close proximity ($<100$ nm) to the nuclear membrane (Figure 2B,C). Further work is needed to clarify the nature of these vehicles involved in intracellular trafficking of C225-HAuNSs. As shown by our simulation study, nuclear membranes having the NIR absorber HAuNS localized within the effective photothermal distance ($<100$ nm) might be capable of mediating significant biological effects upon irradiation with 15 ns laser pulses.

Figure 4. Representative microphotographs of A431 cells irradiated without and with PEG-HAuNS followed by shots of laser pulses. The second laser pulse was instituted at 25 s after the first laser pulse. (A) Without prior treatment with PEG-HAuNS, no apparent morphological changes were noted in cells after two shots of laser pulses. (B) Cells treated with PEG-HAuNS showed occasional nonspecific attachment of PEG-HAuNS clusters to the membrane of A431 cells (left panel, red arrow). After two shots of laser pulses, cells with PEG-HAuNS attached to the cell surface showed membrane blebbing (blue arrowheads). Most cells did not show cellular uptake of nanoparticles. No morphological changes were observed for these PEG-HAuNS-treated cells after two shots of laser pulses (right panel).
positive trypan blue staining and membrane blebbing. observed after two shots of laser pulses (Figure 4B, right panel). However, for most PEG-HAuNS-treated cells that did not have nonspecific attachment to the cell membrane (2 of 40 cells in the field of view, or 5%). After the first shot of a laser pulse, the cells with clusters of PEG-HAuNSs attached to the cell surface membrane disappeared, accompanied by membrane blebbing (Figure 4B, left panel). However, for most PEG-HAuNS-treated cells that did not have nonspecific attachment, no apparent change in cellular morphology was observed after two shots of laser pulses (Figure 4B, right panel).

Figure 3 shows representative microphotographs of A431 cells after treatment with C225-HAuNS followed by irradiation of one and two laser pulses. Before laser irradiation, the viable cells showed cellular accumulation of C225-HAuNS clusters, which were greenish under white light illumination due to NIR absorption. Immediately after the first laser pulse (within 1 s), nanoparticles turned brownish, indicating melting and collapse of the hollow spherical structure of HAuNSs. Interestingly, microphotographic images captured the formation of microbubbles that subsequently collapsed within seconds. The second laser pulse shot 25 s later also induced the formation of microbubbles owing to the presence of the remaining intact HAuNSs in the cells. The nature of these microbubbles is not known. However, they were most likely water vapor bubbles. Within the time frame of less than 1 ns, the temperature in a confined space of nanometer radius could increase rapidly to vaporize water molecules upon interaction of a laser pulse and nanoparticles under high laser intensity. In support of this model, others also observed the formation of water microbubbles while producing laser pulses in the presence of NIR light-absorbing nanoparticles as confirmed photothermolysis. Parameters that would influence the thermal diffusion properties after laser irradiation include laser pulsewidth, laser power density, and optical properties of the nanoparticles. The rate of confined photothermolysis in cells is presumably extended from Arrhenius law, which is used to model cell damage in hyperthermally-induced photothermolysis. We conclude that C225-HAuNS mediates effective cell killing upon irradiation with one or two 15 ns laser pulses.

The cellular environment around the nanoparticle possessed a longer thermal relaxation time than that of metallic nanoparticles; therefore, the fast transient temperature elevation reached a point of vaporization rapidly and the temperature increase was estimated to be confined to a region in a sub-micrometer scale of organelle structures if the laser pulse was short enough. The high magnitude of transient temperature increases in a spatially confined region, even if after only a single pulse, induced significant cellular damage. We refer to the phenomenon of cell damage induced by short laser pulses in the presence of NIR light-absorbing nanoparticles as confined photothermolysis. Parameters that would influence the thermal diffusion properties after laser irradiation include laser pulsewidth, laser power density, and optical properties of the nanoparticles. The rate of confined photothermolysis in cells is presumably extended from Arrhenius law, which is used to model cell damage in hyperthermally-induced photothermolysis.

Figure 5 shows representative microphotographs of A431 cells and 3T3 fibroblasts treated with C225-HAuNS followed by one shot of laser pulse. (A) Representative microphotographs of A431 cells showing extensive trypan blue colorization and membrane blebbing. (B) Representative microphotographs of NIH/3T3 cells showing moderate trypan blue uptake with no morphological change. (C) Quantitative analysis of cells with positive trypan blue staining and membrane blebbing.
temperature elevation and selective cell destruction. These results indicate that irradiation with continuous-wave (CW) laser is ineffective in disrupting C225-HAuNS–treated cells.

To investigate the selectivity of cell damage after shots of pulsed laser, we cocultured A431 cells and 3T3 fibroblasts and treated these cells with C225-HAuNS under the same conditions as before. Under the microscope, these two types of cells had different morphological features. Fibroblasts were bipolar or multipolar with elongated shapes, whereas A431 cells were more cycloid and water drop shaped. A431 cells in the coculture system also had greenish spots revealing binding and internalization of HAuNS clusters. No clusters of HAuNS in 3T3 cells were observed.

To facilitate visualization of membrane integrity, the cocultured cells were shot with a single laser pulse in the presence of trypan blue, a dye widely used as an indicator of membrane disruption. We observed selective and extensive coloration and membrane blebbing after the laser shot over a course of 55 s (Figure 5A). In contrast, no membrane blebbing or morphology change was observed for the fibroblasts within the irradiated field. Unlike that of A431 cells, the cytoplasm of 3T3 fibroblasts had no coloration, although a light blue color in the cell nuclei could be visualized (Figure 5B). Quantitative analysis based on 360 A431 tumor cells showed that 97 ± 5% of these cells were stained by trypan blue dye at 55 s after one shot of laser pulse. Nearly half of treated A431 cells (46 ± 23%) had membrane blebbing. Only 3 ± 5% of treated cells did not show apparent morphological changes and trypan blue staining, possibly, because of a lack of C225-HAuNS uptake in those cells (Figure 5B). Analysis of 78 cocultured 3T3 fibroblasts showed light trypan blue staining in 94 ± 5% of these cells at 55 s after one laser pulse shot; none of these cells had apparent morphological change. About 8 ± 7% 3T3 cells were not affected by the laser pulse (no coloration or membrane blebbing) (Figure 5C). Moreover, the uptake of dye in 3T3 cells was much less and much slower compared with the uptake in A431 cells (Video S1, Supporting Information). Trypan blue stain, which is based on it being negatively charged, does not interact with cells unless the membrane is damaged. Coloration and membrane blebbing of C225-HAuNS-treated A431 cells suggest that one shot of laser pulse was sufficient to cause irreversible membrane damage to these cells. The nature of uptake of the dye in the nuclei of 3T3 cells remains to be characterized. It is possible that this was caused by nonspecific attachment of a small number of HAuNS nanoparticles to these cells and subsequent transient membrane disruption upon laser irradiation.

The observed high efficiency of the cell killing can be attributed to several factors. First, internalized C225-HAuNSs (or other NIR absorbers), which can be attached to the cell membrane or internalized and transported to be in close contact with vital cell organelles (e.g., cell nuclei in the case of C225-HAuNS), are capable of inducing rapid disruption of membrane integrity, leading to exceptionally rapid cellular damage. Second, according to our theoretical modeling, the ns-pulsed laser generates a sharp increase in temperature, which is confined to an area of approximately 0.1 μm in diameter. This means that treatment with a pulsed laser could be highly selective, provided that nanoparticles are delivered to the target cells with high selectivity.

Unlike that with a CW laser beam or other thermal ablation techniques such as radiofrequency ablation, it is possible to confine cell damage with a pulsed laser with a high spatial and temporal control. This notion is supported by our earlier in vivo studies on mice bearing 4T1 breast tumor, in which we used a beam expander to control the illumination size to be ∼4 mm in diameter and irradiated tumors with the 1064 nm pulsed laser at a power density of 4.32 W/cm² for 30 s. We observed a sharp boundary (∼200 μm) separating the laser-ablated zone and the nonablated zone, indicating that treatment with a short nanosecond pulsed laser is feasible and could produce excellent control in ablation margin. Therefore, a pulsed laser might be used as a complement to surgery, in situations in which tumors are located close to vital organs (major blood vessels, nerves, etc.). With regard to potential clinical translation, the Ti:sapphire laser used in our experiments provides nanosecond laser pulses in the NIR region of 700–960 nm. As such, the NIR light pulses generated from the tunable laser are 5 times less powerful than their pumping source (i.e., Q-Switched Nd:YAG laser at 1064 nm), which is widely employed in esthetic and medical applications. We do not anticipate fundamental limitations for moving this type of laser to the clinics. Using the industrial safety enclosure design with an optical articular arm, the laser beam can be safely delivered to the treatment volume in a surgical/interventional oncology suite.

Our data highlight future research opportunities. First, the nature of the blebs shedding from the cells and mechanisms of cell death need to be further defined. Second, the selectivity of cell killing is directly related to the selectivity of cell uptake of NIR-absorbing nanoparticles. The relative contribution of membrane-bound versus internalized nanoparticles to cell damage remains to be determined. In addition, future work is needed to further develop innovative nanoparticles with low nonspecific cellular uptake in nontarget cells and the potential therapeutic applications of short-pulsed laser in selective ablation of tumor cells or tumor-associated stromal cells in clinically relevant animal models of solid tumors. Finally, the laser pulsewidth is an important consideration when studying the Au nanoparticle–laser interaction. For example, Schomaker et al. described the use of femtosecond laser pulses for enhancing siRNA transfection with low thermal impact and minimal effect on cell viability. On the other hand, Ogunyankin et al. showed that for nanobubble generation gold nanoshells prepared from silver nanoparticle templates could be heated to the melting point of gold–silver alloy (~1050 °C) at the laser fluence of 5–20 mJ/cm² with picosecond laser pulses. Therefore, further theoretical and experimental studies are needed to clarify the effect of laser pulsewidth on localized temperature and consequent cellular damage in the presence of gold nanoparticles.

3. CONCLUSIONS

In conclusion, we report highly efficient cell killing by a single short laser pulse. This work suggests that confined photothermalism mediated by targeted NIR light-absorbing nanoparticles is a promising, highly selective treatment modality.

4. EXPERIMENTAL SECTION

4.1. Materials. We performed our studies using hollow gold nanospheres (HAuNSs) that absorb NIR laser light. Poly(ethylene glycol)-coated HAuNSs (PEG-HAuNSs) were obtained from Ocean Nanotech (San Diego, CA) and anti-EGFR antibody-coated HAuNSs (C225-HAuNSs) were synthe-
sized according to the reported procedures.\(^4\) The peak absorption of HAuNS was 780 nm, and the hydrodynamic sizes (average diameter) of PEG-HAuNS and C225-HAuNS were 95 and 173 nm, respectively, as measured by dynamic light scattering. Trypan blue was obtained from Sigma-Aldrich (St. Louis, MO). Figure S2 compares extinction spectra of PEG-HAuNS, C225-HAuNS, trypan blue dye, and culture medium.

4.2. Transmission Electron Microscopy. C225-HAuNS-treated A431 cells were washed and fixed with a solution containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h. After fixation, the samples were washed in 0.1 M cacodylate buffer, post-fixed with 1% buffered osmium tetroxide for 30 min, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were washed several times in water and then dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. The samples were polymerized in a 60°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome. For ultrastructural observation, the samples were fixed with a solution containing 2% osmium tetroxide for 30 min, and stained en bloc with 1% uranyl acetate. The samples were then covered with a cover glass (Fisher) before laser irradiation and microscopic observation.

Representative microphotographs of cells treated with CW laser, extinction spectra, experimental setup to monitor the impact of laser pulses on cancer cells (PDF) Video for visualization of trypan blue uptake in A431 tumor cells and 3T3 fibroblasts after one shot of 15 ns laser pulse (AVI)

4.3. Experimental Setup. The experimental setup is shown in Figure S3. A wavelength-tunable pulsed Ti:sapphire laser pumped by a Q-switched Nd:YAG laser was used for the photothermal experiment. The laser system provides laser pulses with a 15 ns pulsewidth and wavelength options of 1064 and 532 nm and is continuously tunable from 700 to 960 nm. A laser wavelength was selected to match the absorption peak of the nanoparticle applied in each experiment. The laser beam was adjusted by a beam expander and then directed onto live tumor cells seeded in microplates, which were placed under a Leica microscope (Leica Microsystems, Wetzlar, Germany). The images were acquired using a cooled CCD camera (CoolSNAPPro, Media Cybernetics, Rockville, MD). The incident laser energy density of the sample was measured by a pyroelectric energy meter (PE25-C, Ophir, North Logan, UT). The laser energy density was regulated by controlling either the laser pulse energy or the laser wavelength. The laser energy density of the sample was then monitored using a JEM 1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using an AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

4.4. Sample Preparation. A431 epidermoid carcinoma cells overexpressing EGFR and NIH/3T3 fibroblast cells were obtained from the American Type Culture Collection (Manassas, VA). To prepare the cell culture for observation using a microscope, microscope slides (Fisher) were placed into plastic Petri dishes. The cell cultures were incubated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO\(_2\). Twenty-four hours after plating, the medium in the dish was replaced with fresh medium, followed by the addition of either PEG-HAuNS (control) or C225-HAuNS to a final concentration of 2 OD (optical density at 780 nm, ∼0.06 mg Au/mL). After incubation at 37°C for 2 h, the cells were washed two times using the same cell medium to remove unbound HAuNSs before laser treatment. The microscope slides were then taken out of the Petri dish, with a film of medium solution remaining on the surface. The slides were then covered with a cover glass (Fisher) before laser irradiation and microscopic observation.

The authors declare no competing financial interest.

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