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TAT peptide-functionalized gold nanostars: enhanced intracellular delivery and efficient NIR photothermal therapy using ultra-low irradiance

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Abstract

Gold nanoparticles have great potentials for plasmonic photothermal therapy (photothermolysis). However, their intracellular delivery and photothermolysis efficiency have yet been optimized. Here, we show that TAT-peptide functionalized gold nanostars enter cells significantly more than bare or PEGylated nanostars. Their major cellular uptake mechanism involves actin-driven lipid raft-mediated macropinocytosis, where particles primarily accumulate in macropinosomes but may also leak out into the cytoplasm. Following 4-hour incubation of TAT-nanostars on BT549 breast cancer cells, photothermolysis was accomplished using 850 nm pulsed laser under an irradiance of 0.2 W/cm², which is lower than the maximal permissible exposure of skin. These results demonstrate the enhanced intracellular delivery and efficient photothermolysis of TAT-nanostars hence a promising agent in cancer therapy.

Keywords

Nanotechnology; gold nanostars; cell penetrating peptide; photothermolysis; two-photon photoluminescence

Nanoparticle systems have gained wide attention due to their potentials in medicine, such as molecular imaging, immunization, theranostics, and targeted delivery/therapy.^{1–7} Nanoparticles can be fabricated as strong contrast agents for different imaging modalities with superior signal-to-noise ratios than conventional agents,⁸ or as therapeutic agents such as drug carriers,^{9,10} radioenhancers,¹¹ and photothermal transducers.¹² By manipulating the surface chemistry, nanoparticles gain enhanced pharmacokinetics from improved cellular delivery, prolonged serum half-life, reduced immunogenicity/toxicity, and targeted accumulation in specific tissues (e.g. tumor).^{9,13,14} Gold nanoparticles (AuNPs), with their

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Supporting Information. Detailed experimental procedures, additional figures and videos. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

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facile synthesis and biocompatibility, have therefore been applied for a variety of therapeutics, especially in cancer therapy.^{15,16}

Gold nanostars (NS), which feature tunable plasmon bands in the near-infrared (NIR) tissue optical window,¹⁷ bring forth potential for *in vivo* imaging and therapeutic applications.^{18–20} Previously, metal nanoparticle imaging has required the use of fluorescent labels, which are generally quenched on the gold surface. Other non-fluorescent optical tracking methods, using dark-field or differential interference contrast, are typically inoperable in tissue samples.^{21,22} Gold NS, with their unique plasmon resonating with the NIR incident light, creates a non-linear field enhancement that yields intense two-photon photoluminescence (TPL). Their extremely high two-photon action cross section (*e.g.* 10^6 GM), which is several orders of magnitude higher than that of organic fluorophores, allows both *in vitro* and *in vivo* real-time NS tracking without the use of fluorescence.^{19,20,23,24} The ability to visualize NS with high temporal and spatial resolution under multiphoton microscopy provides a tremendous flexibility in understanding nanoparticle kinetics/trafficking behavior in biomedical settings.

Moreover, with a high absorption-to-scattering ratio in the NIR and multiple sharp edges favorable for heat generation, NS efficiently transduce photon energy into heat for hyperthermia therapy.^{20,25,26} To date, most photothermal studies utilize laser irradiation higher than the maximal permissible exposure (MPE) of skin by ANSI regulation.²⁷ To make photothermal applicable to real practice, one needs to enhance the photothermal transduction efficiency. One way is to use a pulsed laser instead of a continuous-wave laser, permitting efficient photothermal conversion by allowing additional time for electron-phonon relaxation.^{12,23,28} Previously, *in vitro* photothermal using NIR pulsed laser reported irradiances of 1.5–48.6 W/cm²;^{23,29,30} which were higher than the MPE of skin (*e.g.* 0.4 W/cm² at 850 nm). Insufficient intracellular particle delivery and low photothermal transduction efficiency may be the main obstacle. Therefore, there is a strong need to design a more efficient photothermal transducer for pulsed lasers (*e.g.* gold NS) with optimized cellular uptake. In this letter, we present TAT peptide-functionalized gold NS for both enhanced intracellular particle delivery and efficient *in vitro* photothermal using a NIR femtosecond laser under an irradiance of 0.2 W/cm², which is lower than the MPE of skin.

To achieve successful and selective photothermal, nanostars need to be delivered sufficiently to the designated target cells without compromising cells' viability. This requires overcoming several biological barriers. For example, particles need to be physiologically stable (*i.e.* non-aggregated, long serum half-life), bind to the cell surface, and traverse the plasma membrane.^{31,32} In general, nanoparticles' size, shape, surface charge, and coating (*e.g.* protein corona, polymer, anti-fouling layer) all affect their cellular delivery.^{33–35} People have tried numerous methods to increase the uptake of nanoparticles. One of the most efficient ways to do this is achieved by surface coating with cell penetrating peptides (CPPs).³¹

CPPs, with 30 or less amino acids that are cationic or amphipathic in nature, facilitate the translocation across the cellular membrane. Human immunodeficiency virus type 1 (HIV-1) encoded TAT peptide, which is one of the most studied CPPs, has been employed to

facilitate not only the intracellular delivery of various nanoparticles,^{36–38} but also the crossing of the blood-brain barrier.^{39,40} It has been shown that TAT-labeled proteins and quantum dots (QD) enter cells by lipid raft mediated macropinocytosis,^{41,42} which is a particularly enticing uptake pathway in drug delivery because of the large uptake volume, avoidance of lysosomal degradation, and the ease of escaping from macropinosomes due to their inherent leakiness.³² TAT functionalization on NS could therefore bring forth enhanced intracellular delivery, which in turns allows efficient photothermalolysis with lower irradiance. To date, although an enhanced cellular uptake of TAT-labeled gold nanoparticles (TAT-AuNPs) has already been observed,^{33,36,43–46} the cellular uptake mechanism for TAT-AuNP remains unreported. We will therefore employ TAT-functionalized nanostars (TAT-NS) as a model system to study their cellular uptake mechanism and temporal profile.

TAT-NS fabrication and cellular uptake imaging analysis

TAT-NS were synthesized as illustrated in Figure 1a. To fabricate stable TAT-NS that resist aggregation in physiological environment and multiple washing cycles, cysteine-terminated TAT peptide (cTAT) and thiolated polyethylene glycol (SHPEG) were both used. We noticed that adding cTAT to NS before adding SHPEG leads to early aggregation. A reverse sequence,²¹ by adding cTAT into PEGylated NS (PEG-NS), resulted in stable TAT-NS (Figure S1, supporting information). The ζ -potential increased from -25.5 mV (PEG-NS) to -17.6 mV (TAT-NS). Enhanced intracellular delivery of TAT-NS (Figure 1b) further confirms the presence of TAT on NS.

Figure 1b clearly shows the enhanced intracellular delivery of TAT-NS, which can be easily visualized under TPL microscopy with high spatial resolution. The cellular uptake of TAT-NS may differ between cell lines.²² Here we use the BT549 breast cancer cell line as a model to demonstrate the enhanced particle delivery. For the first time, the intracellular distribution of TAT-NS, PEG-NS, and bare-NS was investigated and compared on both transmission electron microscopy (TEM) and TPL imaging. On TEM images, numerous TAT-NS are either clustered in vesicles or scattered in the cytoplasm (Figure S2, supporting information). This corresponds to the diffuse white pattern seen on the TPL image (Figure 1b). Because the two-photon axial point-spread-function for a 20 $\times$ water objective is around $1.7\text{ }\mu\text{m}$,⁴⁷ each TPL image may constitute an optical thickness of more than 20 ultrathin sections (~ 70 nm). For example, 100 NS seen on a TEM image correlates to ~ 2000 NS on a TPL image. This could explain why TAT-NS are nearly “saturated” inside cells on TPL image. Meanwhile, TAT-NS were observed in the nuclear region on TPL imaging. However, upon examining several cells on TEM, we did not find any true intranuclear TAT-NS, except some particles in the nuclear cleft, which still appeared to be in the cytoplasm. This agrees with recent studies showing intranuclear localization of smaller TAT-functionalized nanoparticles of 50 nm or less.^{45,46,48,49} The mismatch between TPL and TEM images suggests that intracellular particle distribution characterization using optical methods should be confirmed by TEM. In agreement to previous studies,^{16,33} PEGylation only resulted in minimal cellular uptake at this particle concentration. Also, bare NS without any protective layer tend to aggregate in the vesicles, forming large dense spots on TEM image, corresponding to big white punctates on TPL images. Comparing these 3 surface modifications (TAT, PEG, bare), TAT functionalization greatly facilitates the uptake of gold

nanostars. In the following paragraphs, the uptake mechanism, temporal profile, and cytotoxicity will be addressed.

The cellular uptake mechanism of TAT-NS

TAT peptide operates by anchoring on the plasma membrane and translocating primarily via macropinocytosis, which refers to the formation of large endocytic vesicles of irregular sizes and shapes, generated by actin-driven invagination of the plasma membrane.³² It's been shown that TAT peptide, through multidentate hydrogen binding from arginines (not lysines) with the anionic groups on the membrane (e.g. heparan sulfate proteoglycans, filamentous actin), generates membrane deformation and cytoskeleton reorganization (e.g. actin ruffling) to translocate either directly through membrane or endocytosis.⁵⁰ TAT functionalized proteins or quantum dots also enter cells via macropinocytosis.^{41,42} However, this process has yet to be properly characterized on TAT functionalized gold nanoparticles. We therefore applied both TEM and TPL imaging to assess TAT-NS' intracellular trafficking pathway.

In figure 2b TEM images, numerous TAT-NS are seen bound to the membrane. The binding was not homogeneous throughout the membrane, but formed a patchy distribution; possibly as a result of heterogeneous distribution of heparan sulfate proteoglycans associated with lipid rafts. Figure 2b also shows the surface ruffling in the process of forming a large macropinosome to take up TAT-NS. The ruffling is a common behavior in macropinocytosis that is induced upon stimulation.³² In addition, the vesicle sizes in figure 2b are around 500 nm, which is greater than a typical vesicle size for clathrin-mediated (100–150 nm) or caveola-mediated (50–60 nm) endocytosis. In agreement with Kreptic *et al.* and Berry *et al.*, some particles are observed outside the vesicles in the cytoplasm;^{45,46} this may reflect particles leaking out from macropinosomes into the cytoplasm. All these structural features are in concordance to the behavior of macropinocytosis.

To further assess the TAT-NS internalization pathway, cells were pretreated with several inhibitors for 30 min, incubated with TAT-NS for an hour, then examined under TPL microscopy (Figure 2c) following a previous protocol.^{51,52} We have found that the TAT-NS internalization was inhibited by 4 °C (energy blockade), amiloride (AMR; lowering submembraneous pH), cytochalasin D (cytoD; F-actin inhibition), and methyl- β -cyclodextrin (M β CD; lipid raft inhibition), but not chlorpromazine (CPM; clathrin inhibition), genistein (GNT; caveola inhibition) and nocodazole (NCZ; microtubule disruption; data not shown). This confirms that the TAT-NS internalization is an energy dependent, actin-driven, and lipid raft mediated macropinocytosis, which is in agreement with the findings from Wadia *et al.* and Ruan *et al.* on TAT-protein and TAT-QD, respectively.^{41,42} The clathrin or caveola, although previously were reported on TAT facilitated uptake,³¹ may play a less significant role in this cell type. TAT-NS adhesion to the plasma membrane and actin ruffles, however, were not inhibited because the multidentate hydrogen binding is not affected by the inhibitors. Based on the TEM/TPL results and inhibitor studies, we believe that the primary TAT-NS uptake pathway is through actin-driven and lipid raft-mediated macropinocytosis.

Assessment of temporal cellular uptake profile and cytotoxicity of TAT-NS

Before the photothermolysis study, we need to ensure a sufficient intracellular TAT-NS delivery without compromising the cell viability, so we examined the temporal uptake profile along with the cytotoxicity assay. Figure 3a shows the time-dependent uptake of TAT-NS on BT549 cells. In 10 min, TAT-NS started anchoring onto the plasma membrane. Real-time live cell TPL imaging confirmed the surface binding by showing single free-moving TAT-NS adhering inhomogeneously to the surface membrane (Video S1, supporting information). Within an hour, intracellular uptake can be seen, forming larger-sized punctates on TPL images. These large bright punctates, with sizes around 1 μm on TPL microscopy, were most likely macropinosomes. Smaller and dimmer punctates might be smaller vesicles or even single NS. Later, TAT-NS accumulated towards the perinuclear region and eventually “saturated” the cytoplasm with numerous large bright punctates at 24 hours. Incubation of TAT-NS for 72 hours showed similar particle density as in 24 hours (data not shown). Under TEM, these large bright punctates on TPL imaging were seen to be mostly TAT-NS accumulated in vesicles (Figure S3, supporting information). Krpetic *et al.* also observed particles accumulation mostly in the vesicles at 24 hours, but particles were cleared after replacing the growth medium.⁴⁵ The fate of TAT-NS after 24 hours was not examined in this study. It is possible that macropinosomes interact little with endosomal compartments and may recycle their contents back to the extracellular space,^{31,45} but a detail discussion is beyond the scope of this article.

Figure 3b illustrates that the cellular metabolic activity became affected by TAT-NS after 24-hour incubation (figure 3b). Such effects depend on both the coating type (bare, PEG, TAT) and particle concentration (figure 3c). At 8-hour incubation, the cell viability is not significantly different from the control (0 hr), however the statistical distribution of viability is wide. Although a higher particle density under longer incubation is desired for higher photothermolysis efficiency, to reduce the confounding effect from altered cell viability we chose 4-hour TAT-NS incubation for the photothermolysis study.

In vitro validation of TAT-NS facilitated photothermolysis

The photothermolysis was performed on the same multiphoton microscope with raster scanning for 3 minutes (Figure 3d). The average irradiance (*i.e.* the power density) was controlled by the acoustic-optic modulator and the scanning area from the microscope's software. Here, at 1 mW (12.5 pJ per pulse; irradiance: 0.4 W/cm²), no laser-induced damage was seen on cells treated 4h with media only or PEG-NS. Irradiating cells immersed with PEG-NS (0.1 nM) also did not produce damage (data not shown), most likely because the free-floating PEG-NS were not concentrated enough in cells. In contrast, a distinct square of ablation (empty area) was observed when irradiating (0.4 W/cm²) cells incubated 4 hours with TAT-NS. Real-time live cell TPL imaging showed cells shrinking or moving outwards upon irradiation (Video S2, supporting information). At 0.5 mW (6.25 pJ per pulse; irradiance: 0.2 W/cm²), a large portion of cells were damaged (showing red) but still attached on the dish. Such irradiance (0.2 W/cm²) is not only lower than previously reported values using a pulsed laser,^{23,29} but also lower than the MPE of skin to laser irradiation (0.4 W/cm² at 850 nm) by ANSI regulation.²⁷ This is the first demonstration of cellular

photothermal therapy at such a low irradiance. With longer incubation time (more NS inside cells), the required irradiance could be even lower (Figure S4, supporting information). Combination of pulsed laser irradiation and enhanced intracellular delivery of TAT-NS clearly brings forth a very efficient photothermolysis system.

Note that our TAT-NS have TAT and PEG coating exhibiting beneficial properties from both of them. For *in vitro* study, PEGylation prevent cellular uptake of nanoparticles, hence requires the addition of TAT. For *in vivo* study, the task is even more challenging that requires the consideration of circulating half-life, reticuloendothelial system clearance, vascular permeation, active targeting, and others.^{1,5,53–55} PEGylation extends the nanoparticles' circulation half-life hence promotes the passive accumulation of nanoparticles in the area of enhanced vascular permeability (e.g. large tumor). For regions with normal vascular permeability or even limited permeability (e.g. small tumor), PEGylation may not be sufficient. That may explain why in some cases intratumoral injection is a preferred route for photothermal therapy.⁵⁵ As described in the introduction, TAT-functionalization may facilitate the crossing of blood-brain barrier (BBB);^{39,40} BBB blocks the permeation of many chemicals and nanoparticles. Whether TAT-NS facilitates the accumulation of nanoparticles in the brain tumor remains to be studied. Such discussion is beyond the scope of this paper.

Our results demonstrate an efficient photothermolysis at an ultralow irradiance (0.2 W/cm^2), which is the lowest value ever reported for pulsed laser powers. The enhanced intracellular delivery of TAT-NS substantially potentiates the photothermolysis efficiency without compromising cell viability. The traceability of NS under multiphoton microscopy greatly simplifies both the study of particle's intracellular trafficking and the monitoring of photothermolysis process on live cells. Since multiphoton microscopes utilize tissue penetrating NIR laser, a potential for photothermolysis on deep-seated tumors is possible. Combining NS and TPL microscopy also makes it possible for mechanistic understanding on particle's kinetic behavior. TAT-NS uptake examined on both TEM and multiphoton microscopy confirms that their uptake mechanism involves primarily actin-driven lipid raft-mediated macropinocytosis. Future research would extend this work to selective delivery of cargo to target tissues (e.g. tumors). With further development, gold nanostars can be a promising theranostic agent in cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Kievit FM, Zhang M. Adv Mater (Weinheim Ger). 2011; 23:H217.
2. Shi J, Votruba AR, Farokhzad OC, Langer R. Nano Lett. 2010; 10:3223. [PubMed: 20726522]

3. Farrell D, Alper J, Ptak K, Panaro NJ, Grodzinski P, Barker AD. ACS Nano. 2010; 4:589. [PubMed: 20175564]
4. Chadwick S, Kriegel C, Amiji M. Adv Drug Delivery Rev. 2010; 62:394.
5. Riehemann K, Schneider SW, Luger TA, Godin B, Ferrari M, Fuchs H. Angew Chem Int Ed Engl. 2009; 48:872. [PubMed: 19142939]
6. Wang X, Yang L, Chen ZG, Shin DM. CA Cancer J Clin. 2008; 58:97. [PubMed: 18227410]
7. Nie S, Xing Y, Kim GJ, Simons JW. Annu Rev Biomed Eng. 2007; 9:257. [PubMed: 17439359]
8. Hahn MA, Singh AK, Sharma P, Brown SC, Moudgil BM. Anal Bioanal Chem. 2011; 399:3. [PubMed: 20924568]
9. Ghosh P, Han G, De M, Kim CK, Rotello VM. Adv Drug Delivery Rev. 2008; 60:1307.
10. Huang L, Liu Y. Annu Rev Biomed Eng. 2011; 13:507. [PubMed: 21639780]
11. Juzenas P, Chen W, Sun Y-P, Neto Coelho MA, Generalov R, Generalova N, Christensen IL. Adv Drug Delivery Rev. 2008; 60:1600.
12. Kennedy LC, Bickford LR, Lewinski NA, Coughlin AJ, Hu Y, Day ES, West JL, Drezek RA. Small. 2011; 7:169. [PubMed: 21213377]
13. Ruoslahti E, Bhatia SN, Sailor MJ. J Cell Biol. 2010; 188:759. [PubMed: 20231381]
14. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nat Nanotechnol. 2007; 2:751. [PubMed: 18654426]
15. Hu M, Chen J, Li Z-Y, Au L, Hartland GV, Li X, Marquez M, Xia Y. Chem Soc Rev. 2006; 35:1084. [PubMed: 17057837]
16. Boisselier E, Astruc D. Chem Soc Rev. 2009; 38:1759. [PubMed: 19587967]
17. Weissleder R. Nat Biotechnol. 2001; 19:316. [PubMed: 11283581]
18. Guerrero-Martínez A, Barbosa S, Pastoriza-Santos I, Liz-Marzán LM. Curr Opin Colloid Interface Sci. 2011; 16:118.
19. Yuan H, Khoury (co-first author) CG, Hwang H, Wilson CM, Grant GA, Vo-Dinh T. Nanotechnology. 2012; 23:075102. [PubMed: 22260928]
20. Yuan H, Khoury CG, Wilson CM, Grant GA, Bennett AJ, Vo-Dinh T. Nanomedicine: NBM. 2012; 10.1016/j.nano.2012.02.005
21. Austin LA, Kang B, Yen C-W, El-Sayed MA. J Am Chem Soc. 2011; 133:17594. [PubMed: 21981727]
22. Tkachenko AG, Xie H, Liu Y, Coleman D, Ryan J, Glomm WR, Shipton MK, Franzen S, Feldheim DL. Bioconjugate Chem. 2004; 15:482.
23. Tong L, Wei Q, Wei A, Cheng J-X. Photochem Photobiol. 2009; 85:21. [PubMed: 19161395]
24. Hutter E, Maysinger D. Microsc Res Tech. 2010; 74:592. [PubMed: 20830812]
25. Van de Broek B, Devoogdt N, D'Hollander A, Gijs H-L, Jans K, Lagae L, Muyldermans S, Maes G, Borghs G. ACS Nano. 2011; 5:4319. [PubMed: 21609027]
26. Baffou G, Quidant R, Girard C. Appl Phys Lett. 2009; 94:153109.
27. ANSI American National Standard for safe use of lasers. Laser Institute of America; Orlando, FL: 2000.
28. Huang X, Kang B, Qian W, Mackey MA, Chen PC, Oyelere AK, El-Sayed IH, El-Sayed MA. J Biomed Opt. 2010; 15:058002. [PubMed: 21054128]
29. Au L, Zheng D, Zhou F, Li Z-Y, Li X, Xia Y. ACS Nano. 2008; 2:1645. [PubMed: 19206368]
30. Kim J, Park S, Lee JE, Jin SM, Lee JH, Lee IS, Yang I, Kim J-S, Kim SK, Cho M-H, Hyeon T. Angew Chem Int Ed Engl. 2006; 45:7754. [PubMed: 17072921]
31. Patel L, Zaro J, Shen W-C. Pharm Res. 2007; 24:1977. [PubMed: 17443399]
32. Khalil IA, Kogure K, Akita H, Harashima H. Pharmacol Rev. 2006; 58:32. [PubMed: 16507881]
33. Lévy R, Shaheen U, Cesbron Y. Nano Rev. 2010; 1:4889.
34. Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Proc Natl Acad Sci USA. 2008; 105:14265. [PubMed: 18809927]
35. Bartczak D, Muskens OL, Nitti S, Sanchez-Elsner T, Millar TM, Kanaras AG. Small. 2012; 8:122. [PubMed: 22102541]

36. Torchilin VP. *Adv Drug Delivery Rev.* 2008; 60:548.
37. Wei Y, Jana NR, Tan SJ, Ying JY. *Bioconjugate Chem.* 2009; 20:1752.
38. Zhao M, Kircher MF, Josephson L, Weissleder R. *Bioconjugate Chem.* 2002; 13:840.
39. Rao KS, Reddy MK, Horning JL, Labhasetwar V. *Biomaterials.* 2008; 29:4429. [PubMed: 18760470]
40. Tian, X-h; Wei, F.; Wang, T-x; Wang, D.; Wang, J.; Lin, X-n; Wang, P.; Ren, L. *Mater Lett.* 2012; 68:94.
41. Wadia JS, Stan RV, Dowdy SF. *Nat Med.* 2004; 10:310. [PubMed: 14770178]
42. Ruan G, Agrawal A, Marcus AI, Nie S. *J Am Chem Soc.* 2007; 129:14759. [PubMed: 17983227]
43. Pallaoro A, Braun GB, Moskovits M. *Proc Natl Acad Sci USA.* 2011; 108:16559. [PubMed: 21930955]
44. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R. *Nat Biotechnol.* 2000; 18:410. [PubMed: 10748521]
45. Krpetic Z, Saleemi S, Prior IA, Sée V, Qureshi R, Brust M. *ACS Nano.* 2011; 5:5195. [PubMed: 21609028]
46. Berry CC, de la Fuente JM, Mullin M, Chu SWL, Curtis ASG. *IEEE Trans Nanobioscience.* 2007; 6:262. [PubMed: 18217618]
47. Durr NJ, Weisspfennig CT, Holfeld BA, Ben-Yakar A. *J Biomed Opt.* 2011; 16:026008. [PubMed: 21361692]
48. Pan L, He Q, Liu J, Chen Y, Ma M, Zhang L, Shi J. *J Am Chem Soc.* 2012; 134:5722. [PubMed: 22420312]
49. Panté N, Kann M. *Mol Biol Cell.* 2002; 13:425. [PubMed: 11854401]
50. Mishra A, Lai GH, Schmidt NW, Sun VZ, Rodriguez AR, Tong R, Tang L, Cheng J, Deming TJ, Kamei DT, Wong GCL. *Proc Natl Acad Sci USA.* 2011; 108:16883. [PubMed: 21969533]
51. Zhang LW, Monteiro-Riviere NA. *Toxicol Sci.* 2009; 110:138. [PubMed: 19414515]
52. Iversen T-G, Skotland T, Sandvig K. *Nano Today.* 2011; 6:176.
53. Lal S, Clare S, Halas NJ. *Acc Chem Res.* 2008; 41:1842. [PubMed: 19053240]
54. Fang J, Nakamura H, Maeda H. *Adv Drug Deliv Rev.* 2011; 63:136. [PubMed: 20441782]
55. Huang X, Peng X, Wang Y, Wang Y, Shin DM, El-Sayed MA, Nie S. *ACS Nano.* 2010; 4:5887. [PubMed: 20863096]

Figure 1.

TAT-functionalized gold nanostars. (a) Synthetic schematics for TAT coating on nanostars. Bare NS was coated with thiolated-PEG to stabilize the NS then with cysteine-terminated TAT. (b) Cellular uptake of 0.1 nM bare nanostars, PEG-nanostars, and TAT-nanostars incubated 24 hours on BT549 cells. Aggregated bare nanostars (red arrows) on TEM image correlate to the white big punctates on TPL image. PEG-nanostars showed no uptake. Endosomal (white arrows) and cytosolic (black punctates) TAT-nanostars on TEM image correlate to the diffuse white pattern on TPL image. N: nucleus. C: cleft. Scale bar: 2 μm . TPL image size: 125 $\times$ 125 μm^2 .

Figure 2.

TAT-NS cellular uptake pathway assessment. (a) TPL image of TAT-NS incubated with BT549 cells for 1 hour under 37 °C without inhibitors. (b) TEM images of TAT-NS in vesicles (black arrows), in cytoplasm (circles), on membrane (white arrow), and upon invagination (red arrow). R: ruffle. M: mitochondria. Scale bar: 500 nm. (c) TPL images of TAT-NS treated cells under different inhibitors. The cellular uptake of TAT-NS was inhibited by 4 °C, cytochalasin D, methyl- β -cyclodextrin, and amiloride but not chlorpromazine and genistein. TPL images size: 50 $\times$ 50 μm^2 . Nuclei are stained blue.

Figure 3.

(a) Time series TPL images of cells treated with TAT-NS (white) showing incremental accumulation. Cytoplasm is stained orange. Image size: $50 \times 50 \mu\text{m}^2$. (b) Time series viability on cells incubated with TAT-NS up to 24 hours, where cells' metabolic activity was slightly affected. (c) Cell viability at 24-hour on NS of different surface coatings (bare, PEG, TAT) and concentrations (0.1–0.3 nM). * $p < 0.05$. (d) Photothermalolysis (850 nm, 0.5 or 1 mW, scanning area $500 \times 500 \mu\text{m}^2$, 3 min) on BT549 cells incubated 4 hours with media only, PEG-NS, and TAT-NS. Live/dead cells are green/red. Image size: $612 \times 612 \mu\text{m}^2$.