Hyperthermic effects of gold nanorods on tumor cells

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Plasmon-resonant gold nanorods, which have large absorption cross sections at near-infrared frequencies, are excellent candidates as multifunctional agents for image-guided therapies based on localized hyperthermia. The controlled modification of the surface chemistry of the nanorods is of critical importance, as issues of cell-specific targeting and nonspecific uptake must be addressed prior to clinical evaluation. Nanorods coated with cetyltrimethylammonium bromide (a cationic surfactant used in nanorod synthesis) are internalized within hours into KB cells by a nonspecific uptake pathway, whereas the careful removal of cetyltrimethylammonium bromide from nanorods functionalized with folate results in their accumulation on the cell surface over the same time interval. In either case, the nanorods render the tumor cells highly susceptible to photothermal damage when irradiated at the nanorods' longitudinal plasmon resonance, generating extensive blebbing of the cell membrane at laser fluences as low as 30 J/cm^2.

Keywords: folate receptor, hyperthermia, imaging, nanorods, nonlinear optical microscopy, plasmon resonance, targeted therapy

Hyperthermia is under consideration currently as a noninvasive approach to cancer therapy, in which biological tissues are exposed to higher than normal temperatures to promote the selective destruction of abnormal cells [1]. Systemic hyperthermia has long been considered as an adjuvant therapy for treating various disease states [2–4] and its clinical potential in cancer treatment is an active area of investigation [5,6]. Hyperthermia has diverse therapeutic effects at both the systemic and cellular level. In the latter, a marked increase in local temperature ($\Delta T > 5$°C) can induce the denaturation of proteins or the disruption of organized biomolecular assemblies in the nucleus and cytoskeleton. Moderate increases in temperature can also sensitize cancer cells to cytotoxic agents by increasing membrane permeability and lowering hydrostatic pressure. In addition, mild hyperthermia can induce the production of heat-shock proteins and other immunostimulants, trigger dysfunctional cellular metabolism and promote the onset of acidosis or apoptosis.

Localized hyperthermia has particular relevance in the treatment of primary tumors and early-stage cancers, although its long-term impact on cancer-patient survival remains to be established. Tumor cells are considered to be more susceptible to hyperthermic effects than healthy cells because of their higher metabolic rates, and numerous clinical studies have demonstrated a marked reduction in tumor size after treatment by localized hyperthermia [3,4,7,8]. Several methods have been developed for the external delivery of thermal energy in a nontopical manner, such as microwave irradiation, radiofrequency pulses and acoustic waves (ultrasound). These are capable of deep-tissue penetration but may require a high fluence because of their diffuse nature, possibly producing undesirable hyperthermic effects in surrounding tissues.

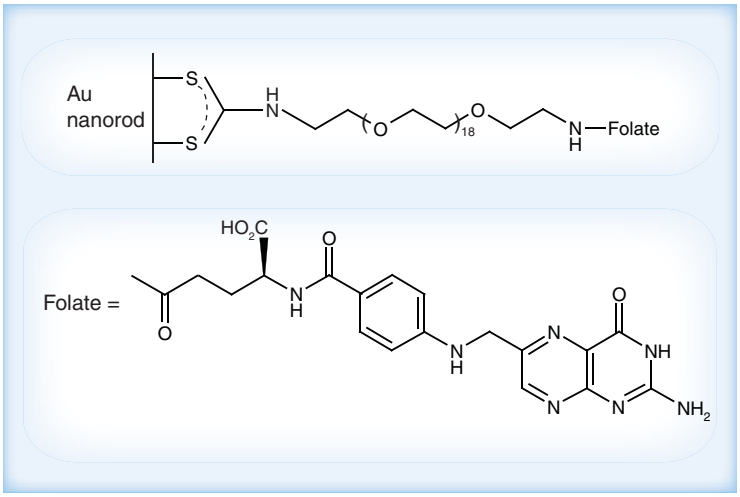
Laser irradiation at near-infrared (NIR) frequencies can penetrate tissues with sufficient intensity and higher spatial precision for inducing localized hyperthermia [9,10], but effective mechanisms are needed to transduce light energy into heat and to discriminate unhealthy from healthy cells [11]. One promising approach is to introduce photothermal agents in the form of anisotropic gold nanoparticles, which can support plasmon resonances with remarkably high absorption cross sections at NIR wavelengths [12–14]. Plasmon-resonant gold nanoparticles are appealing in several respects:

- Their intrinsic toxicity is low;
- Their optical resonances can be optimized for specific NIR frequencies as a function of particle size and shape (for a review, see [15]);
- Their small sizes (<100 nm) are amenable to extravasation and adsorption by diseased cells and tissues;
- Their plasmon-enhanced properties enable them to serve as contrast agents for biological imaging [16–19].

Coincidentally, hyperthermia can promote the uptake of nanoparticles by increasing tissue permeability and blood vessel dilation [2], suggesting a

10.2217/17435889.2.1.125 ISSN 1743-5889

Figure 1. Folate–oligoethyleneglycol ligands conjugated onto nanorod surfaces by *in situ* dithiocarbamate formation.



potential for synergistic effects. The development of plasmon-resonant nanoparticles as multimodal agents for biomedical diagnostics and therapeutics has become an area of intense research activity, with reports from several groups using gold nanospheres [20–22], nanoshells [16,23] and, most recently, nanorods [24,25]. Successful tumor remission in mice has been reported using nanoshell-mediated hyperthermia by NIR irradiation for several minutes, at power densities of only 4 W/cm^2 [26].

Gold nanorods are especially attractive for applications of this sort: they can support a higher absorption cross section at NIR frequencies per unit volume than most other types of nanoparticles and are readily prepared in micellar surfactant solutions using seeded growth conditions [27–29]. The treatment of freshly prepared nanorods with sodium sulfide provides fine control over their longitudinal plasmon resonances and optical stability over time, facilitating their practical application [30]. Nanorods also exhibit significantly narrower linewidths than spherical nanoparticles at comparable resonance frequencies, owing to reduced radiation-damping effects [31], and are also highly efficient at converting light energy into heat, with the highest thermal gradients produced by nanorods embedded in media having low thermal conductivity [32]. We have shown recently that gold nanorods can emit two-photon luminescence (TPL) signals that are sufficiently intense for single-particle detection, owing to their large two-photon absorption cross sections at longitudinal plasmon resonance [18]. This property permits the direct imaging of nanorods in biological samples.

In this article, we image the adsorption and uptake of gold nanorods by individual tumor cells using confocal fluorescence and TPL microscopy and characterize the hyperthermic effects of nanorods upon continuous-wave (cw)-NIR irradiation. We demonstrate that the intense local heating generated by the nanorods can result in extensive damage to the cell membrane, at laser fluences as low as 30 J/cm^2. We also address the critically important issue of nanorod surface chemistry and its role in determining nonspecific cell uptake versus cell-specific targeting, and demonstrate the latter by using folate-functionalized nanorods for directed labeling of tumor cell membranes.

Materials & methods

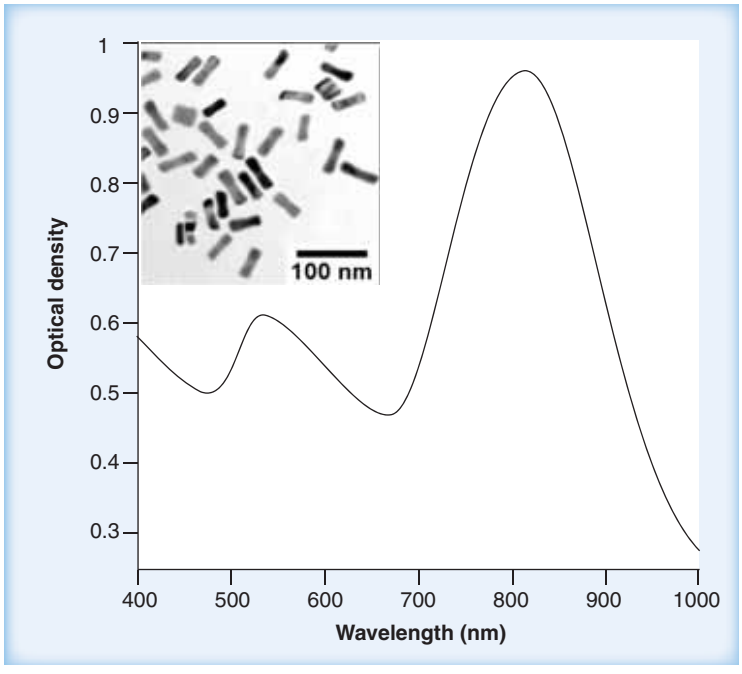
Gold nanorods were prepared in high yields in the presence of cetyltrimethylammonium bromide (CTAB) and silver nitrate using the seeded growth conditions described by Sau and Murphy [28] and were treated with sodium sulfide 15–20 min after injection of the seed solution to arrest their growth [30]. The sulfide-treated nanorods possess a dumbbell-like geometry with slightly flared ends and exhibit a longitudinal plasmon resonance in the NIR that was optically stable over time. The nanorods were centrifuged and redispersed in deionized water twice (24000 g, 5 min per cycle) to remove most of the CTAB and residual metal sulfide. They were then diluted to an optical density in the range of 1.0–1.2.

Folate ligands with oligoethyleneglycol spacers were conjugated onto nanorods by *in situ* dithiocarbamate (DTC) formation, a method developed recently for the robust functionalization of gold surfaces (Figure 1) [33]. A 3-ml suspension of CTAB-coated nanorods was treated with a mixed-bed ion-exchange resin (Amberlite MB-3, Sigma) at room temperature for several hours to remove unassociated surfactant and other ions [34,35]. The nanorod suspension was decanted and treated while stirring with 1 ml of a 10-mM aqueous solution of *O*,*O*'-bis(2-aminoethyl)octadecaethylene glycol (Fluka) adjusted to pH 9.5, followed by 0.1 ml of a saturated (28 mM) aqueous solution of freshly distilled CS_2 to form the DTC anchor. The mixture was stirred for 12 h, then subjected to membrane dialysis for 2 h (MW cutoff: 6000–8000). The amine-coated nanorods were then treated with 0.2 ml of a 10-µM dimethylsulfoxide (DMSO) solution of *N*-hydroxysuccinimidyl folate (NHS-folate) prepared according to a published procedure [36],

followed by additional dialysis to yield a stable dispersion of folate-conjugated nanorods with an absorption maximum at 815 nm and a final optical density close to 1.0 (Figure 2). It is worth mentioning that the displacement of CTAB by the oligoethyleneglycol-DTC ligand resulted in a slight redshift in the nanorod absorption peak, owing to the highly sensitive nature of its longitudinal plasmon resonance to changes in the surface dielectric [30,37].

Adherent human KB cells (a tumor cell line derived from oral epithelium), grown to confluence in serum-free Roswell Park Memorial Institute medium (RPMI), were treated with 100-µl aliquots of nanorod solutions and maintained under standard cell culture conditions at 37°C prior to examination. Nanorod adsorption and cell uptake was monitored by TPL using a laser scanning microscope (FV300/IX70, Olympus). Cells were washed with fresh RPMI growth medium to remove free nanorods prior to TPL imaging, which was performed with a 60× water objective (N.A. = 1.2). A femtosecond Ti:sapphire oscillator (Mira 900, Coherent) operating at 77 MHz was tuned to the nanorods' plasmon resonances (765–820 nm) for optimal two-photon excitation, at an incident power of 1 mW to minimize photothermal damage to the cells or nanorods. Photothermal effects were induced by switching the laser source from pulsed to cw mode.

Figure 2. Extinction spectrum of folate-conjugated Au nanorods with a longitudinal plasmon resonance centered at 815 nm.



Insert: TEM image of nanorods (JEOL 2000FX, 200kV).

Results & discussion

Two-photon luminescence imaging of nanorods in KB cells

Adherent KB cell cultures were exposed to either CTAB-coated or folate-conjugated nanorods and monitored by TPL microscopy. The cells were labeled effectively with nanorods, irrespective of their surface coatings, but major differences were observed in the rate of nanorod uptake. The CTAB-coated nanorods were internalized readily by KB cells and delivered in significant quantities to the perinuclear region after a 5-h incubation at 37°C (Figure 3A) [38]. The TPL contrast provided by the gold nanorods is illustrated by an intensity linescan (Figure 3B). TPL images obtained at different focal depths confirmed that the majority of the nanorods were internalized fully, rather than resting on the cell surface. The cell uptake of CTAB-coated nanorods appears to be nonspecific and is likely to be associated with the formation of endocytic vesicles [38]. The uptake of CTAB-coated nanoparticles by other cell types has also been reported recently [39,40].

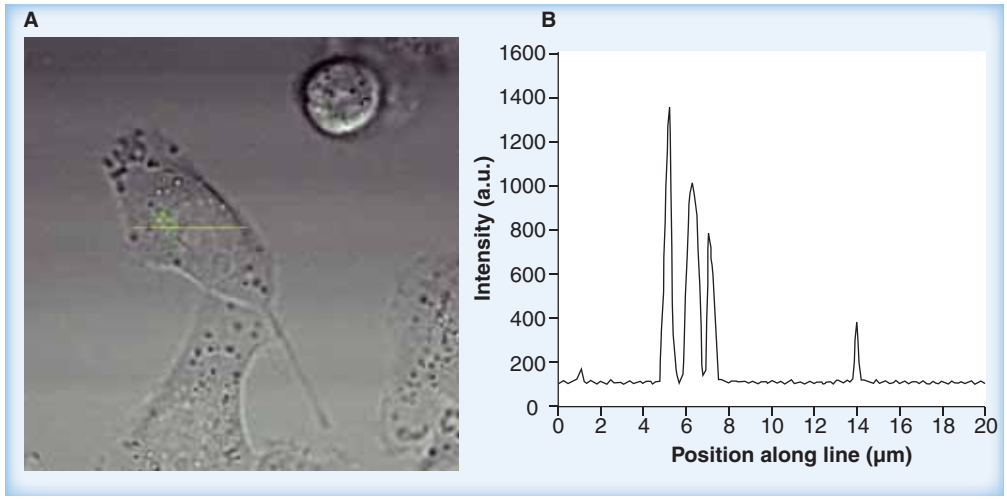
Careful removal of CTAB from nanorods functionalized with folate ligands had a dramatic impact on cell uptake. The folate-conjugated nanorods tended to accumulate on the cell membrane during the first few hours of incubation (Figure 4). Adsorption to the cell membrane is presumably mediated by multivalent binding to cell-surface folate receptors, a widely used system for targeted delivery to tumor cells [41,42]. Although the uptake of folate-conjugated nanorods appears to have been arrested at the cell surface, longer periods of incubation can result in their gradual endocytosis [Tong L *et al.*, Unpublished Data]. The respective roles of CTAB and folate in nonspecific cell uptake and cell-specific targeting are confirmed by some recent observations:

- Nanorods functionalized with methyl-terminated polyethyleneglycol (mPEG) chains are resistant to internalization by KB cells [38];
- Folate-conjugated nanorods are not internalized by NIH-3T3 cells that lack high-affinity folate receptors [Tong L *et al.*, Unpublished Data].

Hyperthermic effects of gold nanorods

We investigated photothermal effects by switching the Ti:sapphire laser from pulsed to cw mode and rastering across regions containing cells of interest.

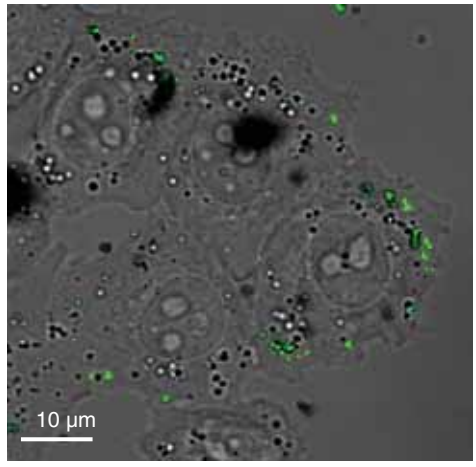
Figure 3. Two-photon luminescence imaging of gold nanorods in KB cells.



(A) Overlay of two-photon luminscence (TPL) and transmission images of cetyltrimethylammonium bromide (CTAB)-coated nanorods internalized by KB cells after a 5-h incubation (linescan = 75 μm). **(B)** Intensity profile across linescan in (A), illustrating the high signal-to-noise ratio provided by TPL contrast.

Designated areas of a culture dish containing KB cells treated with CTAB-coated nanorods were exposed to cw irradiation for 30-s, at fluences ranging from 15 to 120 J/cm^2 (incident laser powers ranging from 7.5 to 60 mW). These cells suffered extensive damage with severe blebbing of their membranes after 30 s irradiation, at fluences as low as 30 J/cm^2 (Figure 5A–F). As a control, untreated KB cells were irradiated for 30 s at 120 J/cm^2, with no visible evidence of hyperthermic injury (Figure 5G & H).

Figure 4. Two-photon luminescence image of folate-conjugated gold nanorods (green) adsorbed to the outer membrane of KB cells after 6 h incubation.

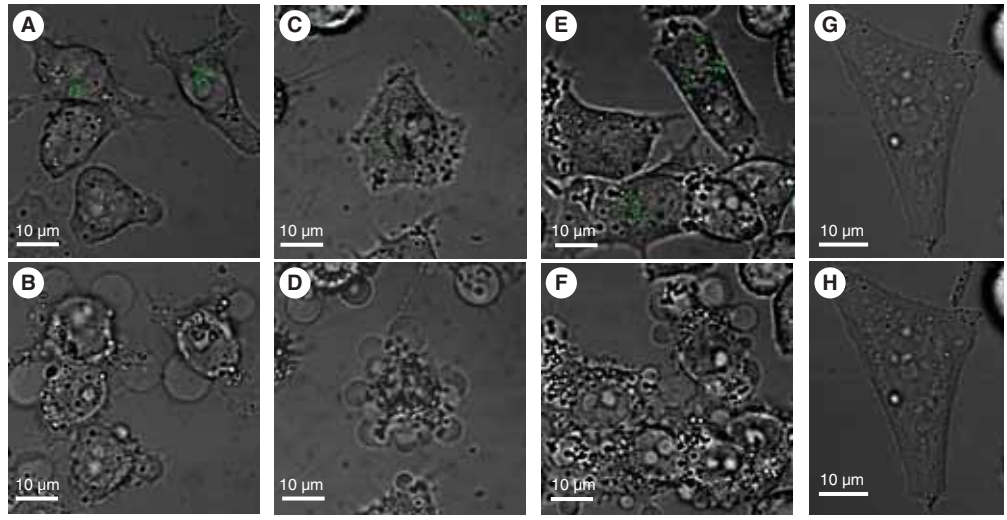


KB cells with internalized nanorods were also evaluated by treatment with ethidium bromide (EB), a fluorescent dye used as an indicator of cell membrane permeability. Cells were treated for 10 min with 2 μl of 0.5 mg/ml EB, then washed and examined by linear fluorescence microscopy (λ_{ex} = 488 nm) as well as TPL microscopy. KB cells containing nanorods were impermeable to EB prior to cw-NIR irradiation but their nuclei were strongly stained after a 30-s exposure, confirming that photothermal treatment compromised the integrity of the cell membrane (Figure 6). Membrane blebbing and nuclear staining was observed only for cells within the region of laser irradiation, confirming the highly localized nature of NIR-induced hyperthermia [11].

It is worth noting that, in addition to the photothermal damage inflicted by the nanorods on the host cells, the TPL signal intensities were greatly reduced or even eliminated completely after cw-NIR irradiation, indicating thermal degradation of the nanorods themselves. Photophysical studies of nanorods using ultrafast laser spectroscopy have shown that an energy pulse in excess of 60 fJ can trigger their structural transformation (melting) into spherical particles, in the absence of an efficient thermal relaxation process [43].

KB cells incubated with folate-conjugated nanorods were also exposed to NIR irradiation and evaluated for photothermal damage. Comparable results were obtained after exposure to

Figure 5. Photothermal treatment of KB cells by cw-NIR irradiation (λ_{inc} = 795 nm, 30 s exposure) at various laser fluences.



KB cells with internalized nanorods (green) before and after irradiation at 120 J/cm^2 **(A & B)**, 60 J/cm^2 **(C & D)** and 30 J/cm^2 **(E & F)**. Severe blebbing of the cell membranes and loss of nanorod TPL signals was observed in all three cases. No visible changes in cell morphology was observed for KB cells without nanorods, before or after irradiation at 120 J/cm^2 **(G & H)**.
cw: Continuous wave; NIR: Near-infrared; TPL: Two-photon luminescence.

cw-NIR irradiation, using similar conditions as described above: the cell membranes again suffered extensive blebbing, accompanied by the loss of TPL signals, indicating the degradation of gold nanorods (Figure 7). This demonstrates that the nanorods do not need to be internalized fully by cells to mediate photothermal damage. Indeed, it is likely that hyperthermic effects can be induced at much lower power densities when the nanorods are on the cell membrane, as this appears to be the region most susceptible to thermal ablation [44]. Further studies are in progress to establish the threshold power needed to inflict nanorod-mediated cell damage.

Conclusion

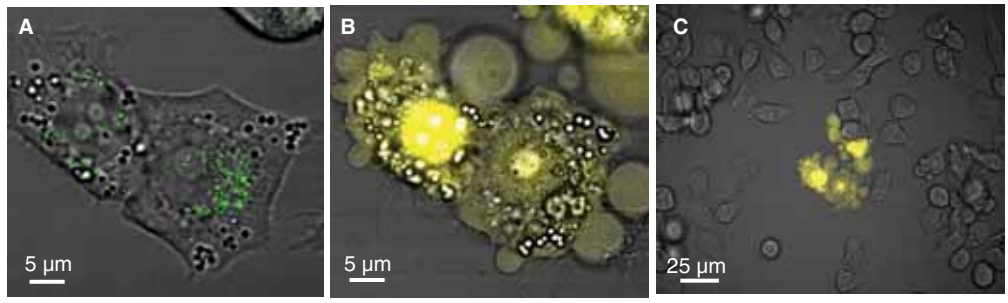
Plasmon-resonant gold nanorods are highly effective at transducing NIR light into heat and are promising as multimodal agents for image-guided therapies based on localized hyperthermia. Nanorod-mediated heating can produce severe blebbing in cell membranes and render them permeable to chemical agents. Nanorods can be targeted directly to the membranes of tumor cells by removing adventitious CTAB to minimize nonspecific cell uptake and by using *in situ* DTC formation for the robust attachment of ligands with high affinity for overexpressed cell surface receptors.

Future perspective

The development of plasmon-resonant nanoparticles into multifunctional diagnostic and therapeutic agents is anticipated to have a great impact on emerging technologies for image-guided therapies. Whereas optical microscopies based on TPL and other multiphoton processes can be used to investigate nanoscale therapeutics in cells and tissues with exquisite spatial resolution, other modalities with greater penetration depth are being developed for *in vivo* imaging. These include optical coherence tomography [17,19], photothermal MRI [16], photoacoustic tomography [45–47] and diffuse optical tomography [48], all of which have already benefited from the application of NIR-active contrast agents.

In order to promote the translational potential of metal nanoparticles as multifunctional agents, one must first surmount the challenges imposed by preclinical evaluations and other outstanding regulatory issues [49]. Colloidal gold particles have been employed as adjuvants in clinical radiotherapies for many years and their approved use provides a welcome precedent. Nevertheless, functionalized gold nanoparticles are regarded as combination products and will require pharmacokinetic testing similar to that of other novel chemical entities (NCEs), namely adsorption, distribution, metabolism and excretion (ADME) profiling. In fact, the recent demonstrations of gold nanoparticles as

Figure 6. Photothermolysis of KB cells mediated by CTAB-coated gold nanorods.



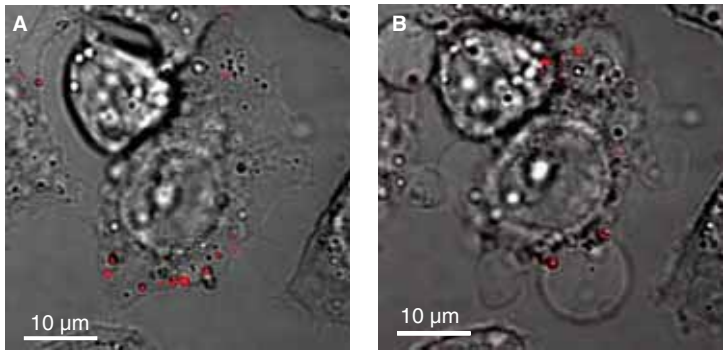
(A) Two-photon luminescence image of KB cells with internalized gold nanorods (green) prior to continuous wave-near-infrared (cw-NIR) irradiation (120 J/cm^2, 30 s exposure). **(B)** Confocal fluorescence image of same cells after cw-NIR irradiation, stained with ethidium bromide (yellow). **(C)** Lower-magnification image demonstrating that photothermal damage to cells is restricted to the region of laser irradiation.
CTAB: cetyltrimethylammonium bromide.

photothermal agents underscore the need for preclinical evaluation, in order to minimize adventitious hyperthermia in healthy tissues.

Stringent control over surface chemistry will be crucial for meeting regulatory standards. In the case of gold nanorods, reproducible ADME characteristics will probably require the rigorous removal of CTAB [38]. Chemically stable coatings are also essential for minimizing opsonization and degradation during targeted delivery. Although thiolated molecules offer a simple and versatile method for functionalizing gold surfaces, they are susceptible to desorption or displacement under physiological conditions [50] and multivalent derivatives, such as thioctic acid, may not fare much better [51]. DTC ligands provide a more robust alternative to thiols for surface functionalization: they have superior resistance to desorption from gold surfaces, yet retain the simplicity of self-assembly [33]. Further studies will reveal whether *in situ* DTC formation can provide a suitable platform for preparing functionalized metal nanoparticles with favorable ADME characteristics.

Although compliance with regulatory guidelines can be a formidable and costly undertaking, efforts are underway to catalyze the translation of potential diagnostic agents from the laboratory into the clinic. In the USA, the US FDA introduced the Critical Path Initiative to remove some of the perceived roadblocks to bringing new medical products to clinical trials, with special considerations given to various forms of biomedical nanotechnology [101]. Last year, the FDA published guidelines intended to accelerate the development of nanoparticles and other NCEs as exogenous contrast agents for imaging. These include guidance on studies for exploratory investigational new drugs (INDs) [102] and on compliance with current good manufacturing practices during Phase I development [103]. A key recommendation in exploratory IND studies is the use of microdosing (i.e., dosages below the pharmacological threshold) for pharmacokinetic assessments. The publication of such guidelines promises to expedite nanoparticle-based INDs into clinical studies on a limited basis, without compromising current standards of health and human safety.

Figure 7. Photothermolysis of KB cells mediated by folate-conjugated gold nanorods.



Two-photon luminescence images of KB cells with membrane-bound gold nanorods (red) after 5-h incubation, before **(A)** and after **(B)** continuous wave-near-infrared irradiation (λ_{inc} = 765 nm, 100 J/cm^2, 30 s exposure).

Acknowledgements

We gratefully acknowledge financial support from the National Institutes of Health (EB-001777–01), the National Science Foundation (CHE-0243496, ECS-0210445), Purdue Research Foundation and a grant from the Oncological Sciences Center at Purdue University. KB cells were kindly provided by Phil Low.

Executive summary

- Plasmon-resonant gold nanorods are strongly absorbing at near-infrared (NIR) wavelengths and have excellent potential for image-guided therapies based on localized hyperthermia.
- Gold nanorods are capable of producing two-photon luminescence, an optical modality with intrinsically low background noise, and can be imaged directly with subcellular resolution.
- Nanorods are highly efficient photothermal transducers and can inflict visible signs of cell injury (membrane blebbing) within seconds of low-intensity NIR irradiation.
- Surface chemistry plays a critical role in the targeted delivery of nanorods with minimal nonspecific cell uptake. Surface functionalization by *in situ* dithiocarbamate formation is a reliable method for conjugating ligands onto nanorods and is sufficiently robust to withstand purification by dialysis.
- Targeted nanorod delivery is demonstrated using folate-conjugated nanorods, which adsorb strongly to the membranes of KB cells for photoinduced membrane blebbing.
- Although safety and regulatory issues remain to be addressed, recent recommendations by the US FDA have created translational opportunities to develop nanorods and other types of nanoparticles as multimodal agents for biomedical imaging and therapies.

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