Chapter 4
Interactions of Gold Nanostars with Cells

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Abstract Unique optical properties, chemical stability, ease of synthesis, and wide functionalization possibilities make GNP attractive candidates for use in biomedical research including chemical sensing, biological imaging, drug delivery, and cancer treatment. In particular, the strong two-photon luminescence of GNP coupled to a specific targeting makes them ideal candidates as contrast agents. To this aim, the interaction with cells and their cellular tracking are important issues for successful application of GNP to biomedical purposes. Properties of gold nanoparticles, namely gold nanostars, as contrast agents and interaction of GNS with cells are highlighted in this chapter.

Keywords Cellular uptake • Intracellular tracking • Contrast agents • Bioimaging

4.1 Intracellular Tracking of Gold Nanoparticles

Rapid advances in molecular biology and genetic engineering provide an unprecedented opportunity for delivery of drugs and genes to intracellular targets [1, 2]. Current intracellular delivery systems are classified as being either viral or non-viral in origin [1]. Viruses are efficient in delivery. However they suffer from poor safety profiles [1, 3]. Non-viral delivery systems, albeit not as efficient as viruses, have the promise of safety and reproducibility in manufacturing [4]. To enhance delivery of drugs and genes to intracellular targets using non-viral delivery systems, it is necessary to have a detailed understanding of the transport process and identify ways of overcoming the cellular barriers [1, 5].

Gold nanoparticles are known to be taken up by cells spontaneously whereby their intracellular distribution depends on many factors such as particle charge, surface modification, particle size and shape, as well as experimental procedures involving concentration and exposure time [6]. Indeed, for biomedical applications,

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surface functionalization of gold nanoparticles is an essential step for any specific targeting of pathological tissues and to allow them to selectively interact with cells or biomolecules [1]. Additionally, for systemic applications, long-circulating nanoparticles are desired both for passive targeting to tumors and inflammatory sites [1] or for specific targeting. Different cellular mechanisms are involved in particle uptake such as phagocytosis and pinocytosis [6]. The latter facilitates the uptake process through small membrane-bound vesicles, called endosomes [6, 7]. Following internalization, membrane-bound vesicles encapsulating the particles mature and eventually fuse with lysosomes [6]. Intracellular gold nanoparticles are trapped inside membrane-bound vesicles of the endocytosis pathway [8]. Trapped in endocytosis vesicles, particles get transported through cells via the common cellular transport mechanisms that relies on the kinesin or dynein molecular motors moving along microtubules that form the intracellular filament network [6, 9]. Studies revealed that average velocities strongly depend on the size of attached cargo that is transported by these motors [10].

With recent achievements in the fabrication of functionalized GNP, which have controllable properties and are decorated with targeting molecules, the endocytosis of such GNPs has become crucial for successful biomedical applications [11]. GNP uptake has employed various cell lines in vitro and GNP of various sizes, shapes, and structures (nanospheres, nanorods, nanoshells, nanocages, nanostars, etc.) [11].

Gold nanoparticles have been considered as potential issues to become versatile biomarkers. For further use of GNP labeled with functionalized molecules, their visualization in biological systems by routine laboratory tools such as light microscopy is urgent.

Nanoparticle contrast agents for molecular targeted imaging have widespread interest in diagnostic applications with cellular resolution, specificity, and selectivity for visualization and assessment of various pathological processes. Single-particle tracking is an important tool to investigate dynamic biological processes by following the movement of individual labeled molecules with high spatial and temporal resolution [12]. Forty nanometer gold nanoparticles are efficient light scatters which have long been used as probes in single-particle tracking application [12, 13]. Moreover, the tracking of individual gold nanoparticles with a spatial resolution of ≈1.5 nm at 25 μs temporal resolution in vivo was demonstrated [14]. As it has already been discussed in previous chapters, excellent optical properties of gold nanoparticles result from the resonant excitation of collective oscillations of the particles' free electrons, known as localized surface plasmons, which cause large optical cross sections at their respective resonance wavelengths. In addition, GNPs are not only very radiant but also display an extreme photophysical stability. Gold nanoparticles do not blink or bleach unlike many fluorescent dyes or quantum dots [12].

Gold nanoparticles can also be treated in order to reduce the cellular toxicity. This fact together with the strong light absorption contributes to make them ideal contrast agents for photoacoustic imaging [15]. On the other hand, gold nanospheres maximally absorb in the visible light region (520 nm), but plasmon coupling following nanosphere endocytosis by cells leads to peak broadening [15].

The unique properties of GNS provide wide opportunities to use as delivery, sensing, and photothermal agents. From an imaging perspective, gold nanoparticles have shown great promise for their use in computed tomography and photoacoustic imaging. Gold nanostars and other gold nanoparticles have recently been examined as contrast agents for biomedical imaging because of their brightness at near-infrared wavelengths, which can penetrate through tissue better than visible light.

Therefore understanding the biodistribution and transport kinetics and the toxicity of gold nanoparticles is vitally important for biological, medical, and pharmacological applications. The following paragraphs focus on GNS as contrast agents and on their interaction with cells.

4.2 GNS Constructs as Contrast Agents for Imaging Applications

For further use of GNP labeled with functionalized molecules, the possibility of visualizing them inside biological systems by means of routine laboratory tools such as light microscopy is crucial.

Nanoparticle-based contrast agents for molecular targeted imaging have in fact widespread interest in diagnostic applications with cellular resolution, due to their specificity and selectivity for visualization and assessment of various pathological processes.

A particularly relevant application of GNS is in the field of microstructural analysis of biomaterials and tissues in ex vivo or in vivo conditions through optical imaging. As it has already been mentioned in previous chapter there are two contributions to the optical properties of the GNS: absorption and scattering.

While GNS with high absorption contribution are more convenient for photothermal or photoacoustic approaches, GNS with high scattering cross section are better for dark-field or Raman-based techniques.

GNS with LSPR in NIR region display greatly enhanced two-photon photoluminescence (TPL). Vo-Dinh and coworkers reported the synthesis of GNS with adjustable geometry particularly suitable for in vivo imaging [16]. The synthesized nanostars showed an extremely strong two-photon photoluminescence process which is confirmed by the quadratic dependence of the luminescence signal up to excitation power (10 mW). The broad-emission spectrum observed by Vo-Dinh et al. implies that TPL from nanostars may originate from electron-hole recombination as it was found in case of nanorods [16, 17], with two cascade excitation processes with a relative delay of only 50–100 ps. The quadratic dependence was not seen on 60 nm gold and silver nanosphere solutions under similar conditions. It was also shown that for higher values of the excitation power the dependence became close to linear. This could be explained by the competition between linear decay and the upconversion for the depletion of the intermediate excited state [18]. Notably, the concentration-normalized emission intensity at 800 nm of nanostar solutions was found to be 1.1×10^4 greater than that of rhodamine B, making the strong

two-photon action cross sections (TPACS) of nanostars more than a million GM units [16].

Gold nanostars can also be applied as strong contrast agents in TPL imaging in biological samples. For this purpose Vo-Dinh et al. reported TPL imaging of wheat-germ agglutinin (WGA)-functionalized gold nanostars on BT549 cancer cells [16], fixed with paraformaldehyde for imaging. The broad-emission spectrum from nanostars resulted in a balanced emission on the red, green, and blue channels resulting in a white color on the reconstructed images. GNS emitted strongly without photobleaching under low laser power (4 mW), which is in the typical working range for organic fluorophores. Unlike gold nanostars, a signal from WGA-coated spheres was observed only under much stronger excitation power.

Tracking of gold spherical nanoparticles in vivo is essential for preclinical studies but was considered as a difficult issue. For fluorescence microscopy one typically needs to label the nanoparticles with organic dyes that are largely quenched by energy transfer to the gold nanoparticles when their mutual distance is <3–4 nm [16]. The labeling of spherical gold nanoparticles requires then the delicate tuning of the distance between the surface of metal nanoparticles and the fluorophores [19]. Alternative techniques able to visualize gold spherical nanoparticles are dark-field and differential interference contrast microscopy. These can visualize gold nanoparticles in cells but not available in tissue.

On the contrary, imaging and tracking of gold nanostars are possible without the need of fluorophores due to strong two-photon action cross sections (TPACS) that is maintained also when the GNS are internalized in cells and in tissues as it has been demonstrated on nude mice [16, 20]. Within 5 min from the injection, PEGylated GNS traveled along the blood vessel where the tissue vasculature became clearly visible at rather low excitation power with minimal tissue autofluorescence background. Due to the high TPACS of nanostars, tracking the motion of PEGylated nanostars in blood vessels was also possible.

In medicine novel techniques with high specificity, such as positron emission tomography (PET), require probe labeling and offer low spatial resolution. Photoacoustic microscopy (PAM) is an emerging imaging modality that combines both rich optical absorption and high ultrasonic resolution in a single-imaging modality [21–23] and it is based on the use of highly absorbance nanoparticles. PAM provides in vivo functional imaging information at clinically relevant penetration depths [21]. Under irradiation of non-ionizing laser pulses, biological tissue absorbs the laser energy and generates heat, resulting in transient thermoelastic expansion and subsequent wideband ultrasonic emission. The produced ultrasonic pressures can be captured by ultrasonic transducers to form photoacoustic (PA) images [21].

Recently GNS have been successfully applied as enhancing agents in PA imaging [24]. Nie and coworkers used a near-infrared pulsed laser as an excitation source and 128 ultrasonic transducers spirally distributed on a hemispherical surface to receive PA signals for the three-dimensional image reconstruction [24]. Cyclic RGD peptide-conjugated plasmonic gold nanostars were designed to specifically target over-expressed integrin $\alpha_v\beta_3$ on tumor neovasculature, enabling highly sensitive angiography by PA and simultaneous photothermal therapy. After the administration

of GNS constructs, tumor angiogenesis was clearly imaged with enhanced contrast and obtained results suggested that PA technique offers deeper imaging depth with homogeneous resolution over existing optical imaging techniques for early diagnosis of tumor angiogenesis.

The use of PAM to monitor the GNS upload kinetics in liver and spleen has been reported recently [21]. Furthermore this approach has been applied to monitor the nanoparticle extravasation to organs from bloodstream in vivo. The structure of the vessel tree and of the internal organs and their changes were clearly imaged by PAM performed at the excitation of 730 nm. The ratio of the PA signal measured in the organ to that measured in the blood vessel was used to quantify the GNS transport kinetics. In order to obtain this, PAM images were continuously acquired every 30 min from a mouse. In parallel, fluorescence images of the mouse at the same time points were acquired to validate the PAM results. The results demonstrate that PAM can potentially provide a noninvasive and semiquantitative method to monitor nanoparticle kinetics in organs and other deep imaging applications where fluorescence instrument cannot be applied.

A unique quintuple-modality theranostic nanoprobe was developed by Vo-Dinh et al. by exploiting SERS, magnetic resonance imaging (MRI), computed tomography (CT), TPL imaging, and photothermal therapy [25]. The synthesized GNS were tagged with a SERS reporter and linked with an MRI contrast agent Gd^{3+}. This nanoprobe exhibited high Gd loading density and superior tumor accumulation due to the enhanced permeability and retention (EPR) effect. These features allowed to reach much higher efficiency MRI and contrast than conventional agents [25]. The relaxivity of Gd-loaded nanoparticles is dependent on several factors, including the intrinsic relaxivity of the linked agent, its local concentration, and the residence lifetime of water protons [25, 26]. Due to the star shape, water protons may effectively interact with the gadolinium chelates and exhibit high proton relaxivity. Moreover, GNS showed extremely high two-photon action cross sections in the NIR region, which makes single-particle detection and real-time particle tracking under multiphoton microscopy feasible. Because corresponding nanoprobes accumulate preferentially in the tumor due to the EPR effect, the highly sensitive TPL optical imaging could be a promising intraoperative tool for tumor demarcation [25] to be compared to fluorescein preoperative staining. Tumor phantom experiments were also performed in this study to demonstrate the potential of GNS constructs for future in vivo applications as labels for CT and MRI. Preliminary measurements showed that the tumor phantom with GNS constructs have higher intensity than ones with GNS-free BT549 cells, therefore making possible to detect smaller tumor phantom than 0.5 cm^3 for early-stage detection [25].

The properties of GNS as excellent contrast enhancement for photoacoustic tomography were also reported by Kim et al. in 2011 [27]. The high photoacoustic sensitivity of GNS at near-infrared wavelengths enabled their in vivo detection in rat sentinel lymph nodes and vessels, with direct application toward lymphangiography.

A gold nanoparticle-Raman reporter-photosensitizer conjugate for optical diagnostic imaging and cancer therapy was reported [28]. As prepared gold nanostars

were multibranched it comprised multiply "hot spots" capable of electromagnetic radiation. GNS were coated with Raman reporter and with hypericin conjugated with denaturated BSA. In vitro studies were performed to examine the uptake of GNS and retention of hypericin's fluorescence upon uptake by the cells. Confocal fluorescence microscopy images showed that nanostars with hypericin concentration of 5 μM can be detected in cytoplasm of the cells and not inside the nucleus. The uptake of GNS within cells and its distribution in the cytoplasm can also be examined using dark-field microscopy, which makes use of the backscattering from the gold nanostars that form core.

Plasmon-resonant nanoparticles are widely used for resonant light scattering, which can be greater by orders of magnitude relative to objects of similar size [29, 30]. These nanoparticles with optical scattering in the NIR are valuable contrast agents for biophotonic imaging and may be detected at the single-particle limit against a dark background. However, their contrast is often limited in environments with high noise. For this purpose gyromagnetic imaging as a dynamic mode of optical contrast was considered, using GNS with superparamagnetic cores [29]. The nanostars exhibit polarization-sensitive NIR scattering and can produce a frequency-modulated signal in response to a rotating magnetic field gradient. This periodic "twinkling" can be converted into Fourier-domain images with a dramatic reduction in background [29]. Gyromagnetic imaging of nanostars inside of tumor cells was demonstrated, using broadband excitation: while their time-domain signals are obscured by incoherent scattering, their Fourier-domain signals can be clearly resolved in less than a second. Notably, gyromagnetically active nanostars do not cause a loss in viability, and even a mild stimulatory effect on cell growth.

Gold nanostars conjugated with cyclic RGD and anticancer drug DOX were studied in different tumor cell lines and in vivo using S180 tumor-bearing mouse model [31]. The weak red fluorescence of DOX in MCF-7 cells indicates that low amount of DOX, Au-DOX, or Au-cRGD-DOX had entered tumor cells. Similarly, the amount of DOX or Au-DOX that entered the MDA-MB-231 cells and Bel-7402 cells was so low that there was no significant difference between the fluorescence intensity from these groups of cells and MCF-7 cells treated with DOX, Au-DOX, or Au-cRGD-DOX. By contrast, Au-cRGD-DOX in MDA-MB-231 and Bel-7402 tumor cells showed obvious red fluorescence. The fluorescence intensity of the three lines incubated was compared. Significant differences appeared in MDA-MB-231 and Bel-7402 cells. All results confirmed that the presence of cRGD on the surface of the GNS increased its cellular uptake by MDA-MB-231 and Bel-7402 cells. The fluorescence images of Au-cRGD-DOX after incubating with MDA-MB-231 cells for 8 h were collected in order to understand the intracellular kinetics of the multifunctional nanoparticles. The obtained data clearly indicated that Au-cRGD-DOX 0r-released DOX entered the nucleus with only a small fraction remaining in the cytoplasm after 8 h incubation and it was confirmed by fluorescence images. In the work by Yuan et al. GNS functionalized with TAT-peptide were also observed in nucleus region by two-photon photoluminescence imaging [32]. These results suggested that the red fluorescence observed from nucleus might be contributed by free DOX released from Au-cRGD-DOX as a result of replacement of glutathione on the GNS surface by abundant intracellular glutathione.

Recent development and application of plasmonic gold nanostars fabricated in by research group led by Vo-Dinh for biomedical imaging has been recently reported [33]. The scientists demonstrated that LSPR significantly increases TPACS to more than a million GM (Göeppert-Mayer) units. TPACS of gold nanostars is greater than that of nanorods and organic fluorophores [16]. The Vo-Dinh research group demonstrated for the first time that GNS with intense NIR contrast under multiphoton microscopy can be visualized in real time [32]. The developed GNS provide a superior nanoplatform for multimodal imaging for cancer diagnostics due to their large surface area for linking other contrast agents like Gd^{3+} and ^{64}Cu, for MRI and PET imaging [25]. GNS with sizes less than 100 nm can accumulate selectively in tumors via the enhanced permeability and retention effect, which is due to the increased leakiness of blood vasculature in tumors [33, 34]. Combining this statement and ability of wide range to be linked with gold nanostars, the last are considered to be suitable platforms for multimodal imaging for cancer diagnostics.

Another promising application of GNS is brain tumor imaging [33]. GNS, as strong optical contrast agents with exceptionally high TPL signal, offer superior flexibility to investigate how drug nanocarriers and contrast agents can be delivered into brain tumor [33, 16, 35]. GNS exhibit longer serum half-life than that of commercial intravascular contrast agents that undergo significant signal decay in less then 30 min. Three hours following systemic injection of PEGylated GNS nanoparticles accumulated preferentially in the tumor than the surrounding area [33]. Moreover, GNS provide a unique nanoplatform for multimodal imaging, which can be used for combined whole-body scans with CT, MRI, or PET and high-resolution optical imaging with SERS and TPL [33, 25]. GNS have also been employed for in vivo tracking with PET imaging [33]. For this purpose GNS were radiolabeled with ^{64}Cu radioisotopes by DOTA chelators linked on the nanoparticles surface. Two-hour continuous PET scan results revealed and immediate nanoprobe uptake in liver. Further studies confirmed that GNS can be tracked in vivo dynamically with sensitive PET imaging.

Another interesting study that showed importance of GNS for biomedical application and particularly for bioimaging was published in *Nanotechnology* in 2012 [36]. PEGylated gold nanoparticles were incubated with melanoma B16-F10 cells. Dark-field microscopy showed that the biocompatible gold nanoparticles were easily internalized and most of them localized within the cells.

A novel class of multifunctional hybrid nanopatches comprised of graphene oxide and gold nanostars for enhanced photothermal effect and image-guided therapy was reported [37]. Internalization of the intact nanopatches into epithelial breast cancer cells was confirmed by Raman imaging, transmission electron microscopy, and inductively coupled plasma mass spectrometry. It appeared that the amphipathic nature and the large surface area of the graphene oxide enable it to serve as a soft, flexible, and biocompatible intracellular carrier for the in situ-grown plasmonic nanostructures and provide long-term biocompatibility with extremely low cytotoxicity. Apart from a remarkably improved photothermal effect compared to that of either of the components at very low dosages of the hybrids and using a low laser power, the hybrid nanopatches exhibit strong Raman scattering, making them excellent candidates for bioimaging, diagnostics, and image-guided therapy applications.

Gold nanostars loaded with high densities of nucleolin-specific DNA aptamer AS1411 produced anticancer effects in a panel of 12 cancer lines containing four representative subcategories [38]. It was found that the nanoconstructs could be internalized by cancer cells and trafficked to perinuclear regions. To visualize uptake by confocal fluorescence microscopy, the 5′-end of Apt was labeled with Cy5 dye prior to attaching the aptamer to the GNS. Light-triggered release further enhanced the in vitro efficacy by making available high local concentrations of Apt near the nucleus. Therefore, it was anticipated that this nanoconstruct can act as a platform for a new class of cell-type-independent agents that could address some current challenges in targeted therapy.

4.3 Interaction of Gold Nanostars with Cells

An increasing number of scientific reports have been published addressing the interactions between nanoparticles and cells as function of their size, shape, and surface chemistry [39–41]. Interactions between nanoparticles and cells should be considered first, since many applications require a strict control over nanoparticle–cell interactions [42]. As it has been already mentioned and explained in previous chapters, gold nanoparticles have attracted particular scientific and technological interest due to their unique optical properties, chemical stability, easy synthesis, and functionalization, all of which make them attractive candidates for use in biomedicine including chemical sensing, biological imaging, drug delivery, and cancer treatment [41]. Indubitably, physicochemical characteristics of gold nanoparticles as they are synthesized and decorated on the surface, significantly affect their circulation, biodistribution, cellular internalization, and trafficking in biological systems [42, 43]. Among all these factors, size and surface's effects on cell interactions have been studied most. For example, Chan et al. have shown that, for spherical gold nanoparticles stabilized by citric acid ligands, 50 nm diameter is an optimal size to maximize the rate of uptake and intracellular concentration in mammalian HeLa cells [44].

As it is discussed in previous subchapter they interaction with cells and in vivo tracking can be easily monitored by applying of various techniques. Direct visualization of interactions between drug-loaded nanoparticles and the cancer cell nucleus was reported [45]. Nanoconstructs composed of nucleolin-specific aptamers and gold nanostars were actively transported to the nucleus and induced major changes to the nuclear phenotype via nuclear envelope invaginations near the site of the construct. The nucleus is known to be the most important organelle in the growth, proliferation, and apoptosis of a cell [46]. Controlling the processes governed by the nucleus has been a primary goal for nuclear-targeted cancer therapy [47]. The number of local deformations could be increased by ultrafast, light-triggered release of the aptamers from the surface of the gold nanostars. Cancer cells with more nuclear envelope folding showed increased caspase 3 and 7 activity (apoptosis) as well as decreased cell viability. It is suggested that this newly revealed correlation between

drug-induced changes in nuclear phenotype and increased therapeutic efficacy could provide new insight for nuclear-targeted cancer therapy [45].

The two most important organelles in drug delivery are the mitochondria and the nucleus. Mitochondria are the powerhouses of cells and key regulators of apoptosis and cell death [48]. Targeting the mitochondria can result in the shutdown of cellular metabolic activities [48, 49]. The nucleus, which possesses genetic material and controls the major biological activities of the cell, unlike the mitochondria, has a membrane surrounding the nucleus—the nuclear envelope, which allows transport of biomolecules via nuclear pore complexes [48]. Small drug molecules can, therefore, enter the nucleus and potentially cause DNA damage and cell cycle arrest [48, 50]. However, the employment nanocarriers for nuclear-targeted therapy faces with main problems, namely lack of understanding on how the cancer cell nucleus and drug-loaded nanoparticles interact at the nanoscale and little control over when the drug will be released from the nanoparticles [48]. Therefore, a new two-component nanoconstruct based on aptamer-loaded gold nanostars that can solve these problems and open new possibilities for future development of nuclear-targeted therapy was reported [48]. This nanoconstruct induced major changes in the nuclear phenotype through nuclear envelope invaginations. Femtosecond, light-triggered release of the aptamers from the surface of the GNS further increased the number of nuclear envelope deformations. Cancer cells with more nuclear envelope folding showed increased apoptosis as well as decreased cell viability. The authors of this paper have revealed that correlation between drug-induced changes in nuclear phenotypes and increased therapeutic efficacy can provide new insight into nuclear-targeted cancer therapy.

In recent publication the same research group described how in vitro efficacy of aptamer-loaded gold nanostars can be enhanced by the increased loading of a G-quadruplex homodimer AS1411 on the GNS surface [51]. In a low pH buffer environment, the loading density of Apt on GNS was increased up to 2.5 times that obtained using the conventional salt-aging process. These highly loaded GNS nanoconstructs were taken up in pancreatic cancer and fibrosarcoma cells ca. two times more and at faster rates compared to normal GNS functionalized with aptamer constructs. When a similar number of GNS carriers was internalized by the cancer cells, the amount of AS1411 delivered via highly loaded GNS nanoconstructs was effectively double. This increased loading of AS1411 enhanced cellular uptake as well as in vitro efficacy of the nanoconstructs in both fibrosarcoma and pancreatic cancer cells. The obtained results suggested that increasing the loading density on GNS could provide a simple means to improve uptake as well as in vitro efficacy of the nanoconstructs in cancer cells.

Vo-Dinh et al. showed that TAT-peptide-functionalized gold nanostars enter cells significantly more than bare or PEGylated GNS [32]. The cellular uptake mechanism involves actin-driven lipid raft-mediated macropinocytosis, where particles primarily accumulate in macropinosomes but may also leak out into the cytoplasm. The results demonstrated the enhanced intracellular delivery and efficient photothermolysis of TAT-GNS constructs, making them promising agents in cancer therapy.

The preparation and properties of GNS modified with biopolymer chitosan were reported [52]. When chitosan was used as capping agent gold nanostars displayed enhanced stability in comparison with gold nanorods. Therefore, they were considered as more suitable mediators in cell photothermolysis because of the slower aggregation and more uniform cellular uptake. Flow cytometry analysis results showed a relatively non-equivalent distribution of chitosan-capped nanorods across the cells in specimen compared with gold nanostars due to highly nonuniform aggregation and cellular uptake of nanorods.

The state of the cells that were treated with chitosan-capped nanoparticles remained mostly unchanged after 72 h, which confirms the biocompatibility of chitosan-modified branched gold nanoparticles. However, the BEAS-2b cell line had a viability below 100 % when the concentration of chitosan-capped GNP was at is highest value (16 μg/mL). This result suggested a concentration limit in the safe uptake of chitosan-capped gold nanostars, which also depends on cell type. Internalization of gold nanoparticles may have an effect other then toxicity on the viability of cells or may disrupt important intracellular processes [52, 53]. The uptake and localization of chitosan-capped GNS by J5 cancer cells were verified by a three-dimensional analysis of the fluorescence distribution via confocal microscopy. The results suggested that GNS in J5 cell would accumulate in cellular organelle, lysosomes.

Shielding nanoparticles from nonspecific interactions with normal cells/tissues before they reach and after they leave tumors is crucial for the selective delivery of NPs into tumor cells. For this purpose and by utilizing the reversible protonation of weak electrolytic groups to pH changes, long-chain amine/carboxyl-terminated polyethylene glycol (PEG)-decorated gold nanostars were designed, exhibiting reversible, significant, and sensitive response in cell affinity and therapeutic efficacy to the extracellular pH gradient between normal tissues and tumors [54]. One PEGylated mixed-charge GNS with certain surface composition exhibited high cell affinity and therapeutic efficacy at pH 6.4, and low affinity and almost “zero” damage to cells at pH 7.4. Remarkably, this significant and sensitive response in cell affinity and therapeutic efficacy is reversible as local pH alternated. In vivo, these constructs showed higher accumulation in tumors and improved photothermal therapeutic efficacy than pH-insensitive GNS.

Surface-enhanced spectroscopic techniques (like SERS and SEF), which rely on local field enhancement, can strongly enhance the spectral intensity of cellular chemical constituents near the particles and serve as tools for ultra-sensitive monitoring of the intracellular distribution of various species [55]. Liz-Marzan et al. presented an effective method to distinguish intracellular from extracellular nanoparticles by selective quenching the SERS signals from dye molecules adsorbed onto star-shaped gold nanoparticles that have nor been internalized by cells [56]. This approach is expected to help understanding actual intracellular nanoparticles distributions. The authors anticipate this localization strategy would provide a means for assessing the internalization efficiency of various cargos coupled with noble metal nanoparticles, such as DNA/RNA, proteins, or drugs which are of major

relevance when studying endocytosis. As it was demonstrated in this paper, it could also prove useful way of checking cell membrane integrity.

Colloidal stable and biocompatible star-shaped SERS-encoded single nanoparticles were prepared and they can be internalized into living cells for intracellular imaging [57]. Encoded nanoparticles, of a final concentration of 6 nM, were incubated with HeLa cells for 2 h at 37 °C. During the internalization by endocytosis, both encoded nanoparticles (stars and spheres) remained separate, without any tendency toward the formation of aggregates inside the endosome. It was assumed that this colloidal stability is related to the adsorption of the cocktail of proteins secreted by the cells. However, semi-quantification of the number of particles per cell showed a higher affinity of spheres to be internalized over stars, around threefold. It was assumed that different affinity must be related with shape, which is in agreement with Cho et al. where spheres were preferentially absorbed over cubes or cages of similar size [58]. Notably, samples exposed to nanostars showed a remarkable SERS intensity while no signal was obtained from those treated with nanospheres even though the number of spheres internalized was considerably larger.

The effect gold nanostars have on the firing rate of neuronal cells have been quantified recently [59]. The evaluation if gold nanoparticles can affect the normal function of neurons, namely their activity and coding properties have been reported recently [60]. For this purpose star-shaped gold nanoparticles of 180 nm average size were synthesized. The authors applied the nanoparticles to acute mouse hippocampal slices while recording the action potentials from single neurons in the CA3 region. The results showed that CA3 hippocampal neurons increased their firing rate by 17 % after the application of gold nanostars. The increase in excitability lasted for as much as 50 min after a transient 5-min application of the nanoparticles. Further analyses of the action potential shape and computational modeling suggested that nanoparticles block potassium channels responsible for the repolarization of the action potentials, thus allowing the cell to increase its firing rate. GNS affected the coding properties of neurons by modifying their excitability.

In the cited in previous subchapter paper published in 2015 it was stated that GNS with the sizes less than 100 nm can accumulate selectively in tumor via well-known enhanced permeability and retention effect, which is due the increased leakiness of blood vasculature in tumors [33]. Therefore, it was found that 70 nm GNS can permeate into the tumor interstitial space but only in close vicinity to tumor vessels 3 h after intravenous injection [33]. Smaller nanoparticles or longer incubation time can further increase the tumor accumulation or extravasation depth [33].

Nanoparticles with one half formed of gold branches and the other of silicon oxide were designed [61]. The oxide face would be used to join the gold nanostars to specific biological receptors that would take them to the damaged cells and the metal part can exercise its therapeutic or diagnostic function.

In this chapter it has been demonstrated that GNS are suitable agents to be monitored in vivo by applying of various microscope approaches. Moreover, it has been briefly discussed the interaction of GNS with cells as this point is considered to be vitally important for successful biomedical application.

References

1. Shenoy D et al (2006) Surface functionalization of gold nanoparticles using hetero-bifunctional poly(ethylene glycol) spacer for intracellular tracking and delivery and delivery. Int J Nanomed 1(1):51–57
2. Torchilin VP, Lukyanov AN (2003) Peptide and protein drug discovery to and into tumors: challenges and solutions. Drug Discov Today 8:259–266
3. Marshall E (2000) Gene therapy on trial. Science 288:951–957
4. Nishikawa M, Hashida M (2002) Nonviral approaches satisfying various requirements for effective in vivo gene therapy. Biol Pharm Bull 25:275–283
5. Kaneda Y (2004) Biological barriers to gene transfer. In: Amiji MM (ed) Polymeric gene delivery: principles and applications. CRC Press, Boca Ranton, FL, pp 29–41
6. Huefner A et al (2014) Gold nanoparticles explore cells: cellular uptake and their use as intracellular probes. Methods 68(2):354–363. doi:10.1016/j.ymeth.2014.02.006
7. Panariti A, Miserocchi G, Rivolta I (2012) The effect of nanoparticles uptake on cellular behavior: disrupting or enabling functions? Nanotechnol Sci Appl 5:87–100
8. Tkachenko AG et al (2004) Cellular trajectories of peptide-modified gold particle complexes: comparison of nuclear localization signals and peptide transduction domains. Bioconjugate Chem 15:482–490
9. Vale RD (2003) The molecular motor toolbox for intracellular transport. Cell 112:467–480
10. Levi V, Gratton E (2007) Exploring dynamics in living cells by tracking single particles. Cell Biochem Biophys 48:1–15
11. Dykman LE, Khlebtsov NG (2014) Uptake of engineered gold nanoparticles into mammalian cells. Chem Rev 114:1258–1288
12. Rong G et al (2008) Resolving sub-diffraction limit encounters in nanoparticles tracking using live cells plasmon coupling microscopy. Nano Lett 8:3386–3393
13. Gelles J, Schnapp BJ, Sheetz MP (1988) Tracking kinesin-driven movements with nanometre-scale precision. Nature 331:450–453
14. Nan XL, Sims PA, Xie XS (2008) Organelle tracking in a living cell with microsecond resolution and nanometer precision. Chem Phys Chem 9:707–712
15. Ricles LM et al (2014) A dual gold nanoparticles system for mesenchymal stem cell tracking. J Mater Chem B 2:8220–8230
16. Yuan H et al (2012) Gold nanostars: surfactant-free synthesis, 3D modelling, and two-photon photoluminescence imaging. Nanotechnology 23:075102
17. Wang DS, Hsu FY, Lin CW (2009) Surface plasmons effects on two photon luminescence of gold nanorods. Opt Express 17:11350–11359
18. Pollnau M et al (2000) Power dependence of upconversion luminescence in lanthanide and transition-metal-ion systems. Phys Rev B 61:3337–3346
19. Huang X et al (2010) A reexamination of active and passive tumor targeting by using rod-shaped gold nanocrystals and covalently conjugated peptide ligands. ACS Nano 4:5887–5896
20. Wang H et al (2005) In vitro and in vivo two-photon luminescence imaging of single gold nanorods. Proc Natl Acad Sci U S A 102:15752–15756
21. Li W et al (2014) *In vivo* quantitative photoacoustic microscopy of gold nanostars kinetics in mouse organs. Biomed Opt Express 5:2679–2685
22. Lee CH et al (2009) Near-infrared mesoporous silica nanoparticles for optical imaging: characterization and in vivo distribution. Adv Funct Mater 19:7688–7693
23. Ye S et al (2012) Label-free imaging of zebrafish larvae in vivo by photoacoustic microscopy. Biomed Opt Express 3:360–365
24. Nie L et al (2014) Plasmonic nanostars: in vivo volumetric photoacoustic molecular angiography and therapeutic monitoring with targeted plasmonic nanostars. Small 10:1585–1593
25. Yang L et al (2013) Quintuple-modality (SERS-MRI-CT-TPL-PTT) plasmonic nanoprobe for theranostics. Nanoscale 5:12126

26. Amie S et al (2007) Gd-loaded liposomes as T1, susceptibility, and CEST agents, all in one. J Am Chem Soc 129:2430–2431
27. Kim C et al (2011) In vivo photoacoustic mapping of lymphatic systems with plasmon-resonant nanostars. J Mater Chem 21:2841–2844
28. Raghavan V et al (2014) Gold nanosensitisers for multimodal optical diagnostic imaging and therapy of cancer. J Nanomed Nanotechnol 5:238
29. Wei Q et al (2009) Gyromagnetic imaging: dynamic optical contrast using gold nanostars with magnetic cores. J Am Chem Soc 131:9728–9734
30. Yguerabide J, Yguerabide EE (1998) Light scattering submicroscopic particles as highly fluorescent analogs and their use as tracer labels in clinical and biological applications: I. Theory. Anal Biochem 262:137–156
31. Chen H et al (2013) Multifunctional gold nanostar conjugates for tumor imaging and combined photothermal and chemo-therapy. Theranostics 3:633–649
32. Yuan H et al (2012) TAT-peptide functionalized gold nanostars: enhanced intracellular delivery and efficient NIR photothermal therapy using ultralow irradiance. J Am Chem Soc 134:11358–11361
33. Liu Y et al (2015) Plasmonic gold nanostars for multi-modality sensing and diagnostics. Sensors 15:3706–3720
34. Gao ZB, Zhang LN, Sun YJ (2012) Nanotechnology applied to overcome tumor drug resistance. J Control Release 162:45–55
35. Yuan H et al (2013) Plasmonic nanoprobes for intracellular sensing and imaging. Anal Bional Chem 405:6165–6180
36. Navarro JR et al (2012) Synthesis of PEGylated gold nanostars and pyramids for intracellular uptake. Nanotechnology 23:465602
37. Nergiz SZ et al (2014) Multifunctional hybrid nanoparticles of graphene oxide and gold nanostars for ultraefficient photothermal cancer therapy. Appl Mater Interfaces 6: 16395–16402
38. Dam DHM et al (2014) Grafting aptamers onto gold nanostars increases in vitro efficacy in a wide range of cancer cell types. Mol Pharm 11:580–587
39. Zhao F et al (2011) Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. Small 7:1322–1337
40. Lewinski N, Colvin V, Drezek R (2008) Cytotoxicity of nanoparticles. Small 4:26–49
41. Coradeghini R et al (2013) Size-dependent toxicity and cell interaction mechanism of gold nanoparticles on mouse fibroblasts. Toxicol Lett 217:205–216
42. Liu X et al (2013) Surface and size effects on cell interaction of gold nanoparticles with both phagocytic and nonphagocytic cells. Langmuir 29:9138–9148
43. Duan X, Yaping L (2013) Physicochemical characteristics of nanoparticles affect circulation, biodistribution, cellular internalization, and trafficking. Small 9:9–10
44. Chithrani DB, Ghazani AA, Chan W (2006) Determining the size and shape of gold nanoparticles uptake into mammalian cells. Nano Lett 6:662–668
45. Dam DH et al (2012) Direct observation of nanoparticles-cancer cell nucleus interactions. ACS Nano 6:3318–3326
46. Zink D et al (2004) Nuclear structure in cancer cells. Nat Rev Cancer 4:677–687
47. Schwartz GK, Shah MA (2005) Targeting the cell cycle: a new approach of cancer therapy. J Clin Oncol 23:9408–9421
48. Dam DH et al (2012) Shining light on nuclear-targeted therapy using gold nanostars constructs. Ther Deliv 3:1263–1267
49. Fulda S, Galluzzi L, Kroemer G (2010) Targeting mitochondria for cancer therapy. Nat Rev Drug Discov 9:447–464
50. Faustino RS et al (2007) Nuclear transport: target for therapy. Clin Pharmacol Ther 81:880–886
51. Dam DH, Lee RC, Odom TW (2014) Improved in vitro efficacy of gold nanoconstructs by increased loading of G-quadruplex aptamer. Nano Lett 14:2843–2848

52. Baginskiy I et al (2013) Chitosan-modified stable colloidal gold nanostars for the thermolysis of cancer cells. J Phys Chem 117:2396–2410
53. Wang L et al (2011) Selective targeting of gold nanorods at the mitochondria of cancer cells: implications for cancer therapy. Nano Lett 11:772–780
54. Wang S et al (2015) Reversibly extracellular pH controlled cellular uptake and photothermal therapy by PEGylated mixed-charged gold nanostars. Small 11:1801–1810
55. Matiajevic E (ed) (2012) Fine particles in medicine and pharmacy. Springer, London
56. Xie N, Lin Y, Mazo M, Chiappini C et al (2014) Identification of intracellular gold nanoparticles using surface-enhanced Raman scattering. Nanoscale 6:12403–12407
57. Rodriguez-Lorenzo L et al (2011) Intracellular mapping with SERS-encoded gold nanostars. Intergr Biol 3:922–926
58. Cho EC et al (2010) The effects of size, shape and surface functional group of gold nanostructures on their adsorption and internalization by cells. Small 6:517–522
59. Kereselidze Z (2014) Interaction of gold nanostars with neuronal cells and single negative terahertz metamaterials with barium titanate resonators. Dissertation, The University of Texas at San Antonio
60. Salinas K et al (2014) Transient extracellular application of gold nanostars increases hippocampal neuronal activity. J Nanobiotechnol 14:31
61. Rodríguez-Fernández D et al (2014) A protecting group approach toward synthesis of Au–silica Janus nanostars. Chem Comm 50:79–81