Au nanostar nanoparticle as a bio-imaging agent and its detection and visualization in biosystems

E. PEREVEDENTSEVA,1,2 N. ALI,3 Y.-C. LIN,1 A. KARMEYAN,1 C.-C. CHANG,1 O. BIBIKOVA,3,4 I. SKOVORODKIN,3 R. PRUNSKAITE-HYYRLÄINEN,3 S. J. VAINIO,3 M. KINNUEN,4,6 AND C.-L. CHENG1,5

1Department of Physics, National Dong Hwa University, Hualien, 97401, Taiwan
2P. N. Lebedev Physics Institute of Russian Academy of Sciences, Moscow, 119991, Russia
3Biocenter Oulu, Infotech Oulu, Faculty of Biochemistry and Molecular Medicine, University of Oulu, Borealis Biobank of Northern Finland, Oulu University Hospital, P.O. Box 8000 FI-90014 Oulu, Finland
4Faculty of Information Technology and Electrical Engineering, University of Oulu, P.O. Box 8000 FI-90014 Oulu, Finland
5clcheng@gms.ndhu.edu.tw
6mattiapanikinnunen@hotmail.com

Abstract: In the present work, we report the imaging of Au nanostars nanoparticles (AuNSt) and their multifunctional applications in biomedical research and theranostics applications. Their optical and spectroscopic properties are considered for the multimodal imaging purpose. The AuNSt are prepared by the seed-mediated method and characterized for use as an agent for bio-imaging. To demonstrate imaging with AuNSt, penetration and localization in different biological models such as cancer cell culture (A549 lung carcinoma cell), 3D tissue model (multicellular tumor spheroid on the base of human oral squamous carcinoma cell, SAS) and murine skin tissue are studied. AuNSt were visualized using fluorescence lifetime imaging (FLIM) at two-photon excitation with a pulse duration 140 fs, repetition rate 80 MHz and 780 nm wavelength femtosecond laser. Strong emission of AuNSt at two-photon excitation in the near infrared range and fluorescence lifetime less than 0.5 ns were observed. It allows using AuNSt as a fluorescent marker at two-photon fluorescence microscopy and lifetime imaging (FLIM). It was shown that AuNSt can be observed inside a thick sample (tissue and its model). This is the first demonstration using AuNSt as an imaging agent for FLIM at two-photon excitation in biosystems. Increased scattering of near-infrared light upon excitation of AuNSt surface plasmon oscillation was also observed and rendered using a possible contrast agent for optical coherence tomography (OCT). AuNSt detection in a biological system using FLIM is compared with OCT on the model of AuNSt penetrating into animal skin. The AuNSt application for multimodal imaging is discussed.

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1. Introduction

One of the advantages of nanoparticles (NP) is a possibility to integrate several functionalities for one nanoparticle-based complexes in biomedical applications. Functionalities such as targeted delivery, controlled drug release, delivery tracking/monitoring, imaging, sensing, and highly localized treatments, can be integrated together to take advantage of the properties of the nanomaterial. Many of these approaches have been implemented with gold nanoparticles (AuNP) [1,2] and they have become extremely popular in biomedical researches and some medical applications [3,4]. AuNP are presented and used in a wide variety of sizes, shapes, and properties. They have been shown to be generally biocompatible [3], their surface can be functionalized to
be conjugated with drugs and other molecules [1–3]. Due to their plasmonic properties, AuNP can be used as an imaging agent [2,4] and in biosensing [1,2]. Additionally, colorimetric sensing, Förster resonance energy transfer (FRET)-based detection, electrical and electrochemical sensing [2] have been demonstrated for different AuNPs. Their applications for photothermal therapy and photodynamic therapy have also been realized [1].

Among AuNPs, gold nanostars (AuNSt) with tip-enhanced plasmonic properties tunable for the near-infrared tissue optical window are especially interesting for biomedical applications in imaging, sensing, and photo-treatment. Based on the plasmonic properties of AuNSt, the applications for optical, photacoustic, and X-ray imaging have been reported [5–7]. AuNSt were applied for sensing with detection by surface enhanced Raman scattering (SERS) [5–8] and plasmonic resonance spectral shifts [9]. AuNSt exhibit a photothermal effect owing to the high molar extinction coefficient in the NIR range [10]. These render AuNSt as suitable agents for photothermal therapy with tunable and adjustable properties [11]. The resonance scattering of visible and near-infrared light upon excitation of their surface plasmon oscillation can be used for optical imaging. In this practice, Au nanostars are used as contrast agents for confocal scanning optical microscopy and for optical coherence tomography (OCT) [12]. In addition, multiphoton-absorption-induced luminescence from Au nanoparticles provides another optical modality for imaging [13,14]. The tip-enhanced plasmonic properties of AuNSt provide possibilities for the control and tuning of spectroscopic properties [13].

Two-photon luminescence from AuNP has been demonstrated to be highly efficient [13] in contrast to the extremely low one-photon luminescence [15]. This is very promising for the detection of macromolecules via enhanced fluorescence [16], Raman [17], cells imaging [18,19], and tissues in-vitro and in-vivo [6,18,20]. Two-photon luminescence of nanostructured gold has been described as a sequential process of absorption of photons following emission as result of the recombination of electrons in the sp-band and holes in the d-band [13]. Lifetimes of different gold nanostructures luminescence can vary in wide range in dependence on shape, size of NP, excitation power and have been shown to be in the ranges 0.25-1 nm [21] and 0.8-2 ns [22]. Generally, the lifetime of fluorescence of many endogenous fluorophores is shorter, thereby providing bio-objects autofluorescence or for dyes used in fluorescence imaging. This provides potential for lifetime imaging (FLIM) [23,24] and FLIM monitoring for the Förster resonance energy transfer effect between gold nanoparticles and fluorescent molecules in biosensing applications [25–27].

In the present work, we focus on fluorescence lifetime imaging at two-photon excitation to visualize AuNSt in biological systems of different structures and thickness to investigate the possibilities of using AuNSt as an imaging agent in bio applications. Although Au nanoparticles have promising potential for bio-imaging and biosensing at two-photon excitation, only a few works concerning two-photon FLIM have been recently reported [28–30] – nanospheres [28–30], nanorods and nanotriangles [29], not Au nanostars. In this work, a cultured cancer cell line (A549 cell human lung cancer cell), tissue (murine skin) and a three-dimensional (3D) cellular model (multi cellular tumor spheroid, MCTS) were interacted with AuNSt. AuNSts are visualized in cell and tissue models using FLIM at a low laser energy. A strong signal at two-photon excited fluorescence, well distinguishable with bio-system autofluorescence and the possibility to keep non-invasive conditions of the experiment (low laser power, low AuNSt concentration) confirm AuNSt’s potential to be used for bio-imaging. The AuNSt’s penetrations into target systems, distribution, as well as possible effects on the samples are discussed. In addition, AuNSt detection in tissue using FLIM and optical coherence tomography (OCT) are compared in the interest of studying their interaction with bio systems and the use for multimodal imaging.
2. Methods and materials

2.1. Au nanostars preparation

AuNSts were synthesized via the reduction of chloroauric acid HAuCl$_4$ in presence of L(+)‐ascorbic acid and silver nitrate AgNO$_3$ at room temperature in aqueous media using presynthesized Au nanospheres as seed [18]. To pre-synthesize, Au nanospheres of 50 nm in diameter, 4.5 mL of 1% sodium citrate was added quickly to 50 mL of boiling 0.03% HAuCl$_4$. For further AuNSt synthesis, 100 µL of the Au nanospheres and 10 µL of 1 M HCl were added to 10 mL of 0.25 mM HAuCl$_4$ water solution at room temperature under vigorous stirring. Then, 100 µL of 2 mM AgNO$_3$ and 50 µL of 0.1 M L(+)‐ascorbic acid were added simultaneously. The solution was stirred for 30 s, then AuNSt were separated by centriﬁge [12,31].

2.2. AuNSt characterization

Size and shape of the particles were estimated with scanning electron microscopic imaging using SEM JEOL (JEOL, Japan). The surface of the AuNSt was analyzed also by Fourier transform infrared spectroscopy (ABB Bomem MB154 FTIR spectrometer, Zurich, Switzerland). The extinction, absorption, and scattering of the AuNSt water suspensions were evaluated from collimated transmittance and diffuse reflectance/transmittance measurements by means of a spectrophotometer system (Optronic Laboratories, USA) with integrating spheres working in diffuse reflectance/transmittance and collimated transmittance regimes. The fluorescence lifetime of the AuNSt at two-photon excitation was estimated. A Ti-sapphire laser, Chameleon Ultra-II (Coherent, USA), was used for the excitation with a wavelength 780 nm; pulse duration 140 fs; repetition rate 80 MHz with laser power 3.5 mW at input to the scanning system. Imaging was performed with a 2D scanner (EINST Technology, Singapore). The signal was detected in spectroscopic range 450-650 nm with a single photon counting system PicoHarp 300 (PicoQuant, Germany) and thermoelectrically-cooled PMT conjugated with microscope Olympus IX71. Objective UPlanFLN 40/0.75 was used. Obtained fluorescence lifetime data were analyzed by a commercially available software package SymPho Time, Version 5.2.4.0 (PicoQuant GmbH, Berlin Germany).

2.3. Biological samples

2.3.1. Cell culturing and AuNSt treatment

The A549 lung carcinoma cell was cultured in a RPMI1640 medium with 10% fetal bovine serum, (Invitrogen Co., USA) in a flask at 37°C and 5% CO$_2$. For further microscopic measurements, the cell monolayer was cultivated on a glass coverslip. The cells were kept in a 6-well plate for two days with the coverslip placed on the bottom (cell concentration was 5 × 10$^4$ per well). The cells were incubated together with AuNSt added in the medium at a concentration of 10 µg/ml at 37°C and 5% CO$_2$ for 2 h. After that, the non-reacting nanoparticles were washed away twice with PBS, and then samples were fixed using 4% paraformaldehyde solution in PBS. The cell samples on glass were investigated using FLIM.

2.3.2. MCTS culturing and AuNSt treatment

The 3D multicellular tumor spheroid (MCTS) was used in this study. To prepare the spheroid human oral squamous carcinoma cell, SAS are seeded in a special well (Gravity TRAP ULA Plate (Insphero)) at a density of 5000 cells per well. SAS cells were cultured in DMEM medium (Gibco, Invitrogen, UK). The medium was supplemented with 2 mM L-glutamine (Invitrogen, USA), 1.5 g/L sodium bicarbonate (Sigma, UK), and 10% fetal bovine serum (Gibco/Life Technologies, Carlsbad, CA, USA). After 3 days of culturing, AuNSt were added into the growth medium (in concentration 10µg/ml), and the MCTS was incubated in the presence of 5% CO$_2$ and 95% O$_2$ at 37°C for 4 days. The spheroid had size of several hundred microns (500-700 µm). The
non-reacting nanoparticles were washed away with PBS and fixed with 3.7% formaldehyde for 24 h. The spheroids grown with AuNSt and without in the growing medium were observed with FLIM.

2.3.3. Skin samples preparation and treatment with AuNSt

The animal care and experimental procedures were performed in accordance with the Finnish national legislation on the use of laboratory animals, the European Convention for the protection of vertebrate animal used for experimental and other scientific purposes (ETS 123), and the EU Directive 86/609/EEC. The animal experimentation was also authorized by the Finnish National Animal Experiment Board (ELLA) as compliant with the EU guidelines for animal research and welfare. Mice of strain Crl:CD1(ICR) of 6-8 weeks age were used for the experiments, provided by Laboratory Animal Center of the Oulu University. Skin patches were obtained and prepared as described in [32].

Six AuNSt treated samples and 4 control samples from 4 different mice were used. Skin samples were approximately 10 mm in size and distributed in wells (Cellstar Tissue culture plates, Greiner Bio-one, Austria). A small chamber was installed and tightly adjoined on the surface of each sample to limit the area of AuNSt suspension treatment. 250 µL of the suspensions with AuNSt concentrations 1 and 0.1 mg/ml in PBS were applied into the chambers. As controls, the same volume of PBS was applied. The skin outside the chamber was covered by a DMEM medium (4.5 g glucose, Gibco, Invitrogen, Dun Laoghaire, Co, Dublin, Ireland) supplemented with 10% of fetal bovine serum (Gibco, Dublin, Ireland) and 1% penicillin–streptomycin (Sigma-Aldrich, St Louis, MO, USA). The skin samples were incubated for 24 h at 33°C and 5% CO₂ and then fixed with 4% paraformaldehyde (PFA) for further optical-spectroscopic analysis. Samples were divided in two parts: one was used for OCT measurements, and the second was cut in cross-sectional slices for lifetime imaging measurements. OCT measurements were done with the samples as-prepared and with cleared by benzyl benzoate and benzyl alcohol mixture (2:1) (Sigma-Aldrich, USA) according to the method previously described [33].

2.4. Optical studies

2.4.1. Fluorescence lifetime imaging

FLIM has been used to visualize the AuNSt in the skin via analysis of lifetime distribution. Setup is described in Section 2.2 AuNSt characterization was used. Laser power (in the input to scanning system) was varied from 3.0 mW used for measurements of samples containing AuNSt to 20 mW for control samples. The excitation wavelength varied in the 760-800 nm range and the signal was collected in the 450-650 nm range.

2.4.2. Optical-coherence tomography

OCT measurements were done using high-speed spectral domain Hyperion OCT imaging system (Thorlabs, Inc., USA) with broadband light source, with a center wavelength of 930 nm, the axial resolution 5.8 µm and the lateral resolution 8 µm in air. The output optical power of the device is below 5 mW and the axial scan rate is 110 kHz. The sample was placed in the Petri dish and covered with thin layer of PBS to avoid dehydration.

2D and 3D OCT images were obtained. 2D images contained 1024×512 pixels and the time of measurement was 2 sec. About 20 images for each sample have been measured. 2D images were used for further analysis of 1D in-depth reflectance profiles (A-scan). To calculate the A-scan, the parts of images without artifact reflection or scattering from hair and surface of PBS were selected. Origin software was used to transfer the image to numerical matrix. Columns of the matrix corresponding to selected parts of the images were used for analysis. The results obtained from several images were averaged for each sample and the standard deviation was calculated.
Averaged or individual A-scans were considered for the analysis of peculiarities and identification of common patterns. 3D images were used to observe the clearing effect.

2.4.3. Confocal fluorescence microscopy imaging

Confocal images were obtained using scanning confocal microscope TCS SP5 (Leica, Germany) to reveal the skin morphology. Laser with a wavelength of 532 nm and objective HC Plan APO 20/0.7 (air) were used.

3. Results and discussion

Figure 1 depicts the characterization of the studied AuNSt. In Fig. 1(a), the SEM image of AuNSt is presented; and it displays the uniformity of the samples prepared. The size of the particles, with a tip-to-tip diameter about 100 nm, their star-like shape had an average of 10-15 tips and were 20-50 nm in length. Our purpose is to analyze the properties of AuNSt in terms of their applications for multimodal imaging. In general, the properties of Au nanoparticles are significantly determined by localized surface plasmon resonance (LSPR), collective oscillation of the free electrons of the conduction band induced by interaction with electromagnetic waves/incident light [34]. Plasmon resonance wavelength depends on the size and morphology of the particles [10,12,18,34]. For gold nanostars, the plasmonic-determined effects on optical (scattering, absorption, emission of light) and thermal properties are determined also, first, by the high curvature on the tips. The high curvature on the tips creates a larger surface charge density and consequently a higher local electric field [5]. Secondly, core-tip localized plasmon hybridization also determines the effects on optical and thermal properties [35].

Initially, AuNSt were considered as nonfluorescent markers for optical bioimaging applications due to the intense LSPR scattering detectable in the differential interference contrast regimes in conventional microscopy [12] and contrast increasing in OCT [31]. However when using multiphoton excitation, photoluminescence efficiency increases, which makes AuNSt a useful fluorescent imaging agent for multiphoton imaging [6–8,22,36]. The process of two-photon excited luminescence in gold nanoparticles has been described [13,15,22]. The first photon excites an electron in an occupied d-band (or from the sp band below the Fermi level) to an unoccupied energy above Fermi level. The second photon excites a d-band electron and recombines with the sp-band hole. Subsequent two-photon excited luminescence is generated. Resonantly excited two-photon excited luminescence can be strongly enhanced by the plasmon resonance [13,22].

The plasmonic resonance absorption of the AuNSt was observed. In Fig. 1(b), absorption, extinction and scattering spectra of AuNSt (dissolved in bidistilled water) are shown. Absorption reveals a plasmon resonance peak centered near 760 nm. Increased scattering of near-infrared...
light upon excitation of the surface plasmon is also observed. Two-photon photoemission of the AuNSt was excited using a femtosecond laser with excitation wavelengths in the range of 760-800 nm. The signal was collected in a broad range, 450-650 nm. Note, the detection in a broad range is not sufficient to discuss the origin of the signal in detail. We mentioned two-photon excited fluorescence like the most studied non-linear optical phenomena arising in AuNSt. This excitation range overlaps with the observed plasmonic resonance peak with the tissue optical window (700-1300 nm) and corresponds also to optimal set-up range. Application in the tissue optical window is important for further applications of AuNSt as a theranostic (imaging, phototreatment) agent in tissues.

Aside for use as an imaging agent for multiphoton microscopy, AuNSt is also suitable for use as a marker for two-photon fluorescence lifetime imaging (FLIM) and time-gate imaging. The fluorescence lifetime for gold nanoparticles have been found to be in the range of 0.25-2 ns [21,22]. The lifetimes were about 2 ns and considered longer than could be expected, probably due to the recombination of electrons located close to the Fermi surface responsible for the observed photoluminescence [22]. However, the fluorescence of gold nanoparticles lifetimes are found shorter compared to most endogenous fluorophores and are well distinguishable for cell labeling. Au nanorods, as a cellular marker for FLIM, have been previously reported in few papers. Au nanorods with fluorescence lifetime in the order of 0.1 ns have been used for marking canine kidney MDCK cells [23]. Nanorods, nanospheres and nanotriangles have been visualized with FLIM in ovarian cells [29]. Gold nanoparticles are also used in conjugates with fluorescent molecular FLIM bio-label or bio-sensor with fluorescence lifetime detection or FRET monitoring to increase label fluorescence [27]. Despite the great potential of gold nanostars, they were not implemented up to date.

With the tip-enhanced properties of AuNSt, their two-photon action cross-section has been shown about two orders of magnitude higher than that of Au nanorods. For other shapes, the difference is even more [6], thus, more intense two-photon luminescence was observed as compared with other kinds of the Au nanoparticles [34]. The fluorescence time decay measured for AuNSt is shown in Fig. 1(c). The estimated lifetime is about 0.5 ns, much shorter than lifetimes usually observed for autofluorescence of biological systems and many fluorescent dyes commonly used for the staining of biological samples. Therefore, one can expect that AuNSt can be reliably detected when interacted with biological samples. It is advantageous that the visualization of AuNSt using FLIM can be combined with studies of the cell or tissue state via analysis of endogenous fluorophores’ lifetimes and distribution. For example, the analysis of nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate redox couples [37] or the analysis of distribution of flavin adenine dinucleotide, elastic fibers, keratin, and others [38] is of particular use in the aforementioned studies. However, while previously using gold nanoparticles as a fluorescent marker, FLIM was demonstrated for cells [23,28,29], we have extended the studies to include tissue models with using Au nanostars.

Human lung carcinoma A549 cells were used to visualize the AuNSt interaction with cell. Previously, AuNSt penetration into 2D cell cultures has been studied. Uptake of gold nanoparticle of different shape was induced via various endocytosis mechanisms, depending on the particle shapes and sizes [39,40]. Clathrin-mediated endocytosis pathway is attributed to the penetration mechanism for AuNSt [39]. Similar results have been observed for AuNSt penetration into growing 3D cell spheroids [41]. However, in general, the interaction of Au nanoparticles with 2D and 3D cell cultures is not quite comparable [41]. Thus, using AuNP for imaging or therapeutic agent studies on tissue models is important to determine their in-vivo behavior.

AuNSt cytotoxicity was analyzed in previous works and was found low [12,31]. In general, cytotoxicity analyzed without light treatment for different kinds of 2D cultured cells has been found depending on both Au particles and cells. Cytotoxicity can be low for some analyzed cells and medium for others [42]. AuNP effects on biological tested objects are dependent on
the NP shape, size, dispersion medium [43,44]. Different NP can affect different structures and processes in the investigated bio-samples [44]. The information about AuNSt cytotoxicity revealed in comparison with other AuNP looks controversial [44,45]. Irradiation of biological samples treated with AuNP by a near-IR laser in continuous mode [41,46] have an observed NP concentration-dependent photokilling effect even at low laser power. This effect is used for photothermal therapy (PTT) combined with imaging applications. All this means is that the NP effects on the target sample have to be evaluated for more concrete measurements taking into account the toxic/disturbing influence or efficacy of photothermal treatment [47]. In the presented work, we are focusing on the imaging ability of AuNSts, thus the most important is that in imaging methods short-pulse laser, such as two-photon imaging or FLIM, are preferable in cases when photothermal effects are undesirable, due to decreasing heating at decreasing pulse duration [48]. Using different kinds of AuNP for imaging in various modifications of two-photon microscopy has been already repeatedly demonstrated [13], while for FLIM very limited number of publications exists [28–30]. Using two-photon microscopy cellular uptake of Au nanorods [49,50] and nanospheres [50] was observed.

FLIM images of cells treated with AuNSt are shown in Fig. 2(b) in comparison with the untreated control cells (Fig. 2(a)). The images of cells treated with AuNSt reveal strong signal with low lifetime, less than 1 ns, characteristic for gold nanoparticles. The images in general show that AuNSt can penetrate into cell and localized in cytoplasm [34,39]. It can be concluded from the characteristic cell shape with the voids in the nuclei areas surrounded by AuNSts, which outline the cytoplasm, are analogous to previously observed cells [50]. The imaging reveals the signal of AuNSt is much stronger than cells autofluorescence; thus it can be excited with a much lower power, which is important for reducing photothermal effects. The AuNSt lifetime is significantly shorter than the lifetime of cell autofluorescence, as a result, AuNSt are well distinguished in the cell images and they can mark the cells. The difference in the values of lifetimes of AuNSt interacting with the cell and cell autofluorescence can be seen when comparing time decay curves and histograms of lifetime distribution for the cell and for the nanoparticles (Fig. 3).

To visualize AuNSt interaction with the tissue model system, scaffold-free 3D MCTS were prepared using oral cancer cells SAS. After 3 days of culturing in the special well, the spheroid has a size of several hundred microns (Fig. 4(a)). Such MCTS were incubated with AuNSt in the growing medium for 4 days, and the spheroids grown with and without the AuNSt are analyzed with FLIM. In Fig. 4, extended microscopic (Fig. 4(b)) and FLIM (Fig. 4(c)) images of MCTS edge are shown.

FLIM is measured at excitation power 20 mW. Autofluorescence intensity and lifetime are in general comparable with these cells in 2D cell culture (Figs. 2 and 3), the time decays of AuNSt,
Fig. 3. Lifetime decays of cell and MCTS treated with AuNS and control cell autofluorescence. Inset: Histogram of distribution of lifetimes along the corresponding images.

AuNS in 2D cultured cell and 3D cultured cell are practically the same. In Fig. 5, the FLIM images of MCTS focused at different positions along the z-axis are shown.

Due to the “intrinsic confocality” of two-photon microscopy the images of different cross-sections can be observed from the upper outer layer (Fig. 4(a)) of the MCTS to inside of the spheroid (Figs. 4(b) and 4(c)). Note, that in contrast to [41] decreasing intensity of signal of AuNS in the MCTS cells at measurements of deeper cross-sections inside the spheroid was not...
observed, probably due to high AuNSt concentration. Thus, when the cells of the spheroid were labeled with AuNSt, the inner area of the spheroid was observable with FLIM using a low laser power (3 mW). Therefore, the power and AuNSt concentration can be further decreased to safer values. Compared to the control, without AuNSt, the only signal from cells on the MCTS surface can be observed (Fig. 4(c)), even with power increased up to 20 mW.

The observed images of AuNSt-labeled cells inside the MCTS also evidenced that in 3D cell culture a significant fraction of the nanoparticles are localized in the cell cytoplasm. AuNSt FLIM can be used to study NP’s ability to penetrate the 3D target of interest and to identify their localization within the cells.

For comparison and analysis of the applicability and possible limitations of MCTS, a second tissue model was used. An interaction of AuNSt with murine skin in-vitro was studied. In general, NP can enter the organism via different pathways, in particular transdermally [51]. Skin interaction with nanoparticles is recently widely studied and analyzed, first, in terms of applications in pharmaceutics and medicine (particularly, using for drug delivery and other treatments) especially when local topical effects are desired [52]. Second, it is studied from the safety point of view. For example, NP which can be contained in cosmetics (sunscreens, etc.) [53] and for industrial and environmental safety concerns of NP [54]. Additionally, current development of nanoparticles for use in theranostic applications, combining drug delivery with imaging and/or treatment requires the understanding the pathways of penetration into skin and mechanisms of interaction [52]. In this respect, mice skin is an ideal tissue model for studies.

Mice skin was treated with the AuNSt suspensions in PBS. Skin morphology was observed with confocal microscopy. To detect AuNSt in the skin samples and to visualize the particles and skin interaction, FLIM and OCT have been applied. The images are presented in Fig. 6. Confocal images at one-photon excitation in Fig. 6(I), of control skin Fig. 6(Ia), and skin treated with AuNSt, Fig. 6(Ib), reveal skin structure with hair follicles and sebaceous glands. Skin structure was identified by comparison with anatomic data [55]. Figure 6(I), both control and AuNSt-treated samples do not reveal AuNSt as they are practically not fluorescent at one-photon excitation as well as are not detectable via NP scattering at a laser wavelength that is lower than plasmon resonance. Two-photon excited FLIM images of mice skin (Fig. 6(II)) also show hair follicles, in addition, stem cells of dermal papilla niche can be observed. No signal which could be attributed to AuNSt is detected in control sample (Fig. 6(IIa)), while images of treated samples (Fig. 6(IIb)) show a strong signal with short lifetime revealing the presence of AuNSt, predominantly in different compartments of the hair follicles. They also can be observed in surrounding tissue. Thus, in addition to transfollicular pathway of NP penetration into skin, which has been repeatedly demonstrated and discussed [56,57], well detectability of the AuNSt with FLIM allows discussion of NP ability to distribute outside follicles into surrounding tissue structures and provides complimentary information with OCT.

This possibility and conditions for nanoparticles to penetrate skin layers and accumulate in certain compartments until recently are not fully studied. Size-dependent penetration of Au NP [58], including AuNSts [59], of different sizes into skin was studied using electron microscopy [58] and microscopic studies of haematoxylin and eosin stained skin sections [59]. Well detectability of Au nanoparticles with FLIM can be useful in further study of this issue. The murine skin ultrastructure consisting of multiple layers mimics human skin. Murine skin offers us a good model for visualization and distinguishing between the signal of the AuNSt, skin autofluorescence and for utilizing AuNSt multifunctional features. Like confocal fluorescence imaging, 2D OCT images also show a not clearly detected and well-interpreted difference between control skin (Fig. 6(IIa)) and the treated skin (Fig. 6(IIb)). However, OCT images allow numerical estimation via analysis of 1D in-depth reflectance profiles or A-scans (Fig. 7) and direct observing the increased contrast after clearing the sample (Fig. 8).
Fig. 6. (I) Confocal microscopic images, objective 20×; (II) FLIM images, objective 40×, 790 nm laser excitation, power 10 mW, signal is detected in the spectral range 450-650 nm; (III) OCT 2D images of mice skin. (a) Control; (b) sample treated with AuNSt (1 mg/ml).

However, OCT images allow numerical estimation via analysis of 1D in-depth reflectance profiles or A-scans (Fig. 7). Figure 7(a) shows in-depth distributions of the backscattering signal for the control sample and samples treated with AuNSt averaged a large number of individual A-scans. The shadow indicates the standard deviation and the observed difference is low for averaged data. It can be explained by the non-homogeneous structure of the skin and by the non-homogeneous distribution of the nanoparticles, so analysis of non-averaged A-scans can provide additional local information. In Fig. 7(b), examples of characteristic individual A-scans are compared for control and samples treated with AuNSt in concentration of 1 mg/ml.

The effect of AuNSt on A-scans is qualitatively observable in individual A-scans, characteristic for control and AuNSt-treated samples, shown in Fig. 7(b), with signal inhomogeneous increasing which can be due to AuNSt concentrating and contrasting on skin layers border [51]. The comparison of scans from control and treated skin reveals the increased contrast in the range of skin thickness about 0.1 mm for treated samples which can be attributed to penetrated AuNSt.
mostly localized at the junction between the different skin layers. This observation is qualitatively comparable to previous reports [60] about in vivo interaction of gold nanoshells with silica core NP of sizes of about 150 nm with rabbit skin (despite significant differences in the experiment conditions and used skin structure).

Dependence of the backscattering of the skin, treated with AuNSt on the AuNSt concentration was also observed. Comparison of averaged A-scans calculated for samples treated with AuNSt of 2 different concentrations is presented in Fig. 8(I). AuNSt applied in high concentrations are clearly affecting the skin backscattering, while A-scans measured for the sample treated with AuNSt with the concentration of 10 times lower are rather comparable with control. This result is confirmed by OCT images of skin after clearing shown in Fig. 8(II). In Fig. 8(IIa), 3D OCT image of skin without clearing is presented for comparison, demonstrating light scattering from skin structures. Cleared samples show scattering predominantly from the surface skin layer and from individual hair follicles which increases when the AuNSt concentration increases.

4. Conclusion

Au nanostars have been considered for multifunctional theranostic applications including multimodal bio-imaging, based on their plasmonic properties enhanced by star-like shape. In this study, we demonstrated and analyzed Au nanostars interaction with cellular and tissue models using FLIM and OCT. The prepared AuNSst reveal plasmonic resonance in near-infrared (NIR) region with wide peak centered at 760 nm. The resonance scattering of NIR light upon the excitation is close to their surface plasmon oscillation allowed the AuNSt to be used as contrast agent for OCT. Strong two-photon excited fluorescence of AuNSt with fluorescence lifetime less than 0.5 ns was observed.

Detection of AuNSt at their interaction with cellular culture, multicellular 3D tissue model and murine skin in vitro using FLIM at two-photon excitation has been demonstrated. AuNSt are well detectable in 2D cell culture and in 3D MCTS tissue model, found localized in cell cytoplasm. The observation of bright FLIM imaging at different layer of the MCTS proved AuNSt are capable of imaging the 3D/thick tissue model volume using low laser power.

In artificial MCTS AuNSt could be considered localized inside cells. The AuNSt interaction with more complicate non-homogeneous real tissue structure was observed using murine skin.
AuNSt were detectable in the skin with FLIM, results of FLIM were also compared with OCT data: calculation of 1D in-depth reflectance profiles and 3D OCT images of cleared tissue, providing enough contrast for analysis. This combination allows study of the AuNSt penetration into and distribution in the skin. AuNSt were detectable in the skin with FLIM, results of FLIM were also compared with OCT data. This combination allows study of the AuNSt penetration into and distribution in the skin. The AuNSt transfollicular pathway of the penetration is confirmed; additionally, well detectability of AuNSt allows their observing in skin structures along-side the hair follicles. We think that using AuNSt as imaging agent can help understanding of processes of nanoparticles’ interaction with tissue in general. Additionally, understanding of AuNSt penetration and localization in skin promotes widening the possibilities of AuNSt multifunctional theranostic applications.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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