Supporting Information

Liposome-Gold Nanorod/siRNA Complexes as a Potential Theranostic Agent using Multispectral Optoacoustic Tomography

Adrian Taruttis[1,2], Neus Lozano[3], Antonio Nunes[2], Dhifaf A. Jasim[3], Nicolas Beziere[1,2], Eva Herzog[1,2], Kostas Kostarelos[3], Vasilis Ntziachristos[1,2]

EXPERIMENTAL

Materials. DOTAP (1,2-dioleoyloxypropyl)-N,N,N-trimethylammonium chloride), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and cholesterol (Chol) were purchased from Avanti Polar lipids (USA). Solvents (chloroform and methanol) were obtained from Fisher Scientific (UK). Scrambled siRNA and apoptotic siPLK1 were purchased from Eurogentec (Southampton, UK). The fluorescence dye NIR-797 (λ_{exc}=795nm, λ_{em}=817nm) was supplied by Sigma Aldrich and used conjugated with DOPE by adding NIR-797 (15mg, 0.017mmol) to a solution of DOPE (32mg, 0.042 mmol) and triethylamine (1mL) in chloroform. The mixture was stirred for two days and controlled by thin layer chromatography (TLC). Evaporation of the solvent was carried out and the resulting residue was purified on a silica column using step gradient elution. Commercially available gold nanorods (AuNR) with peak absorption in the near infrared (Ntracker 30-PM-700/780/850, Nanopartz Inc., Loveland, CO, USA) were employed to add optoacoustic contrast to the nanocarrier system. AuNR display high absorption in the NIR region and tunable longitudinal plasmon resonances dependent on their aspect ratio, and are thus highly suitable as optoacoustic contrast agents.[1, 2]

Preparation of liposomal nanocarriers. Double-labeled NIR-liposome-AuNR hybrids used as a nanocarrier were prepared using NIR797-DOTAP:Chol (0.1:2:1mM) and 475µg/mL AuNR (longitudinal sizes of 29, 38 and 45nm) and engineered by self-assembly following the lipid film hydration and sonication methodology.[3] The triple-labeled NIR-liposome-$AuNR_{780}$/siRNA complexes were formed via electrostatic interactions and obtained by mixing in a 1/1 fashion the siRNA stock solution to the NIR-liposome-$AuNR_{780}$ stock solution, at the charge ratios of 0.4, 0.8, 1.2, 2, 3 : 1 (N/P, positively charged nitrogen from DOTAP to negatively charged phosphate from siRNA).

UV-Vis spectroscopy. The optical absorption spectra of the colloidal solutions were recorder with a Varian Cary winUV 50 Bio spectrophotometer (USA). All samples were diluted 10 times in distilled water prior the measurement.

Agarose gel electrophoresis. Agarose gel 1% (w/v) in TBE buffer was prepared, loaded in gel casting tray and allowed to cool for 30 min at room temperature. Samples were prepared by keeping the final amount of siRNA constant to 0.5µg in order to maintain the signal of

siRNA constant and allow visualization by ethidium bromide (EtBr) stain when exposed to UV light. TBE buffer was added to the cooled gel with EtBr stain. Loading dye was added to samples immediately before loading into the wells. Electrophoresis was run using Bio-Rad (USA) apparatus at 70V and 400mA for 45 min. Images were captured using UV light box (G:BOX) with Gene Snap software.

Dynamic light scattering (DLS) and electrophoretic mobility. Particle diameter (Ø) and electrophoretic mobility (μ) were measured at 25±0.1°C by a Malvern Zetasizer unit, Nano ZS series HT. The particle size is based on dynamic light scattering technique in back scattering mode, at 173° and λ=632.8nm. For μ measurements the same dispersions were placed into U-shaped cuvettes, equipped with gold electrodes. The zeta potential (ζ) is related to the μ by Henry's equation valid in the Smoluchowski approximation, when the screening length is much smaller than the particle radius.

Transmission electron microscopy (TEM). Transmission electron microscope measurements were performed with a Tecnai 12 instrument operated at 120kV accelerating voltage. A drop of the dispersions was placed in formvar/carbon coated copper grids and then stained with 1% UA. The excess of liquid was removed with filter paper.

MSOT imaging system. The custom MSOT system employed acquires, reconstructs and displays 2D transverse slice images through mice at a rate of 10 frames/second.[2, 5] Excitation light is provided by a tunable optical parametric oscillator (OPO) pumped by a Q-switched Nd:YAG laser (Opotek Inc., Carlsbad, CA) with a tuning range in the near-infrared (700-950nm) for maximum tissue penetration. The laser pulse duration is approximately 10ns and the pulse repetition frequency is 10Hz, which governs the maximum frame-rate. The beam is coupled into a fiber bundle assembly (CeramOptic Industries, Inc., East Longmeadow, MA) that is divided into 10 output arms to illuminate the mouse from multiple angles on the imaging plane. A custom-built piezocomposite ultrasonic transducer array (Imasonic SAS, Voray, France) with 256 elements and a central frequency of 5MHz is used for detection. The resulting axial resolution is estimated to be in the region of 190 microns. The elements are arranged in one row forming a spherical concave array covering 270° with a mechanical focal distance of 4cm. The time-resolved signals are digitized in parallel by a custom-built acquisition system with 256 channels. The signals are acquired at 40MSamples/s and 12 bit digital resolution. The transducer array and optical fiber outputs are submerged in a temperature-controlled water bath. Mice are positioned horizontally in a holder with a thin polyethylene membrane so that there is no direct contact between water and the mouse. The laser beams and ultrasonic transducer array are in fixed positions, whereas the animal can be moved through the imaging plane using a linear stage (NRT 150/M, Thorlabs GmbH, Dachau, Germany) to enable imaging of multiple transverse slices. The MSOT images were reconstructed using a filtered back projection algorithm.[6] Following reconstruction of single-wavelength images, Independent Component Analysis (ICA) was applied to identify the distribution of the NIR-liposome-AuNR hybrids by means of their unique absorption spectrum.[7] This data-driven approach separates datasets into maximally statistically independent components.

Phantom imaging. To confirm the ability of MSOT to detect the NIR-liposome-AuNR$_{780}$ hybrids, cylindrical phantoms of 2cm diameter were prepared by molding 1.3% wt of agar powder (Sigma-Aldrich, St. Louis, MO, USA) mixed with 6% of Intralipid 20% emulsion (Sigma-Aldrich) to obtain a background with an a reduced scattering coefficient of μ's ≈ 10cm-1. A cylindrical inclusion with a diameter of approximately 3mm in the center of the cylinder contained a solution of the nanocarriers. Multispectral data sets of the phantom were recorded in the experimental MSOT system between 700nm and 900nm with 10nm steps each using 100 signal averages (acquisition time of 10s per wavelength).

Animal handling. Experimental procedures involving animals were approved by the government of Upper Bavaria. A total of 5 mice were used in this study. *In vivo* MSOT imaging was performed to spectrally differentiate signals generated by the NIR-liposome-AuNR hybrids from background tissue absorption. We employed 4T1 mouse breast cancer and HT29 human colon adenocarcinoma cancer cells; in each case inoculated subcutaneously in adult female CD1 nude mice (Charles River Laboratories, Sulzfeld, Germany). In the case of the 4T1 tumors, 0.8 million cells in 15μl BD Matrigel™ (BD Biosciences, Bedford, USA) were used per mouse. The cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin in an atmosphere of 5% CO2 at 37°C. For the HT29 tumors, 1.5 million cells were used. These cells were cultured in McCoy medium with 1% penicillin/streptomycin and 10% FCS in an atmosphere of 5% CO2 at 37°C. Upon reaching a tumor diameter of approximately 7mm, mice were anaesthetized with 2% Isofluorane and intra-tumorally injected with the NIR-liposome-AuNR$_{780}$/siRNA complex. The injected volume of the complex was 25μl.

MSOT imaging studies. To investigate MSOT imaging of the liposomal nanocarriers, we performed *in vivo* MSOT imaging by recording optoacoustic data at multiple excitation wavelengths (700nm, 730nm, 760nm, 770nm, 800nm, 860nm and 900nm) using 100 averaged signals per wavelength, resulting in an acquisition time of 70s per slice. The wavelengths were selected to be able to resolve the NIR-liposome-AuNR$_{780}$ hybrids from the background absorption of the tissue. Imaging time points were directly before injection, 10 min and 24h after injection. Multiple slices were acquired at 1mm intervals to cover the entire tumor region.

Cryosection imaging. Following *in vivo* MSOT, mice were euthanized by cervical dislocation, embedded in an optimal cutting temperature compound (Sakura Finetek Europe BV, Zoeterwonde, NL) and frozen to -20°C. To serve as validation of the MSOT results, and to determine the distribution of NIR-liposomes, we performed fluorescence cryoslicing imaging (FCSI).[8] Similarly to the MSOT imaging geometry, FCSI sliced the frozen mice in the transversal plane, at a 500μm micron pitch, and recorded color and fluorescence images from each slice. The FCSI system is based on a cryotome (CM 1950, Leica Microsystems, Wetzlar, Germany), fitted with a motorized spectral illumination and multi-spectral CCD-based detection in epi-illumination mode.[8] Fluorescence images were captured at 817nm (peak emission) to resolve the liposomes based on their fluorescent label NIR-797 .

TUNEL staining for apoptosis detection. To confirm apoptosis within the tumors, fresh-frozen tissue sections (thickness 20µm) were processed for immunohistochemistry using DeadEnd™ Fluorometric TUNEL assay (Promega ®). Briefly, free-floating paraformaldehyde fixed 20 μ m sections were treated for 15 min at room temperature (RT) with Proteinase K solution (1:500). After washing with Tris-Buffered Saline and Triton 100x (TBST) solution, the slices were incubated for 10 min with 4% PFA and wash for 5 min with TBS before being equilibrated with Equilibration Buffer at RT for 10 min. TdT incubation buffer was prepared following the Kit User Manual suggested ratios of Equilibration Buffer, Nucleotide Mix (labeled with Fluorescein to stain the fragmented DNA caused by apoptosis), and rTdT Enzyme. The tailing reaction was performed in the dark and humidified chamber (RT) for 60 min. The reaction was terminated by adding 2x SSC and incubating for 15 min at RT in the dark. After two washing steps with TBST for 5 min each, the slices were mounted and sealed on glass slides with hardset Vectashield (containing DAPI to stain cell nucleus). The sections were then imaged with a epi-fluorescent microscope (Leica DM 2500, Leica Microsystems), using the suitable filer-cubes from Chroma Technology Corp (Bellows Falls, USA).

Dark-field microscopy. To confirm the presence of AuNR inside the tumors, tissue samples were characterized using dark-field scattering microscopy. Fresh-frozen tissue sections (thickness 12µm) were positioned on the stage of an upright microscope (Leica DM 2500, Leica Microsystems) mounted with an oil dark-field condenser (NA = 1.2 - 1.4) and a 40x oil objective (Leica Microsystems). Pictures were taken using a color camera (EC3, Leica Microsystems).

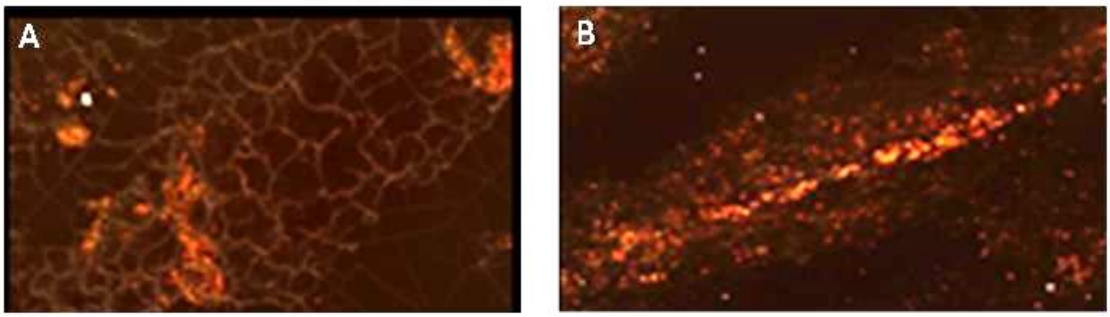

Figure S1. Dark-field microscopy of the corresponding cryosections confirming the presence of AuNRs (red points) in (**A**) 4T1 and (**B**) HT29 tumors 24h after injection of NIR-liposome-$AuNR_{780}$/siPLK1 complexes.

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