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Rapid Communication

# Epidermal Growth Factor Receptor–Specific Nanoprobe Biodistribution in Mouse Models

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ABSTRACT

Nanotechnology offers a targeted approach to both imaging and treatment of cancer, the leading cause of death worldwide. Previous studies have found that nanoparticles with a wide variety of coatings initiate an immune response leading to sequestration in the liver and spleen. In an effort to find a nanoparticle platform which does not elicit an immune response, we created 43 nm and 44 nm of gold and silver nanoparticles coated with biomolecules normally produced by the body, α-lipoic acid and the epidermal growth factor (EGF), and have used mass spectroscopy to determine their biodistribution in mouse models, 24 h after tail vein injection. Relative to controls, mouse EGF (mEGF)–coated silver and gold nanoprobes are found at background levels in all organs including the liver and spleen. The lack of sequestration of mEGF–coated nanoprobes in the liver and spleen and the corresponding uptake of control nanoprobes at elevated levels in these organs suggest that the former are not recognized by the immune system. Further studies of cytokine and interleukin levels in the blood are required to confirm avoidance of an immune response.



## Introduction

Nanomedicines are versatile delivery systems that enable the selective *in vivo* distribution of a wide variety of compounds for both imaging and therapeutics. They increase the localization of therapeutic compounds, including imaging agents and radiosensitizers, targeting tissues such as solid tumors. The successful use of nanomedicines *in vivo* requires a clear understanding of how the properties of the carriers (composition, size, physical structure, etc.) affect their pharmacokinetics and biodistribution.

We have developed[1-2] a nanocarrier, which targets the epidermal growth factor receptor (EGFR), a membrane protein which mediates cell growth, proliferation, and differentiation in multiple tissues. Overexpression of EGFR is a hallmark of many cancers and precancers, including breast, lung, bladder, skin, mouth, brain, and neck.[3-12] The nanocarrier provides an order or magnitude improvement in contrast compared with the existing techniques.[13-14] This study is designed to assess the biodistribution of a modified version of our probes, which have all the necessary properties to serve as a molecular imaging agent.[1]

Previous studies have investigated the effect of size, surface charge, and administration route on nanoparticle biodistribution. Sonavane et al.[15] found that the distribution of gold nanoparticles is size dependent. However, nanoparticles of all sizes were present in the liver, spleen, and lungs. Along with the size of the nanoparticles, the surface charge was also found to be a strong determinant of the biodistribution.[15-16] Zhang et al.[17] found that the level of toxicity associated with high concentrations of some nanoparticles is dependent on the administration routes, with tail vein injection resulting in the lowest toxicity.

Modified EGF has been investigated as a radiotherapeutic agent, *in vivo*, in immune-compromised athymic nude mice–bearing tumor xenografts for which EGFR overexpression is a hallmark. Pharmacokinetic studies conducted by Senekowitsch-Schmidtke et al.[18] found that $^{125}$I-EGF accumulates in breast, colon, and

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gastric xenograft tumors and exhibits rapid blood clearance (half-life of <1 min) leading to tumor-to-blood ratios of 3:1 within 6 h of injection. Bue et al.[19] found tumor-to-blood ratios of 2:1 to 6:1 in xenograft urothelial carcinoma studies after the injection of technecium-99m–labeled EGF-dextran conjugates. Most recently, Li et al.[20] conjugated cysteine tagged EGF with $^{18}$F (N-[2-(4-[$^{18}$F]fluorobenzamido)ethyl]maleimide) and found tumor-to-nontumor ratios of 6:1 and rapid blood clearance (<1 min) as detected by microPET imaging of head and neck squamous cell carcinoma xenografts. In all cases, the rapid kinetics and tracer uptake was thought to arise from the small size of the tracer (~6.4 kDa for EFG) compared with EGFR-specific antibodies. These studies highlight the capacity of an EGFR-specific agent to accumulate at high levels in xenograft tumors in immune-compromised mice. To understand whether our EGFR-specific nanoprobes provide a good platform for future *in vivo* studies in organisms with robust immune systems, we have investigated their biodistribution in immune-competent C57BL/6 mice.

For our study, medium-sized (~40 nm) gold and silver nanoparticles are used. These nanoparticles are produced in concentrations ($10^{11}$ particles per mL ~ 2 pM) that are not expected to produce any adverse effects when administered in 100 μL quantities ($10^{10}$ particles or $10^{-16}$ mol).[21] Gold nanoparticles have been found to interact better with cells, but silver particles scatter light more strongly[22] making the latter ideal for optical sensing applications.

As mentioned earlier, most studies concerning nanoparticle distribution have encountered issues with nanoparticle sequestration by the liver and spleen. We demonstrate here that our nanoprobes are *not* sequestered by the liver and spleen because they have a different biomolecular coating, α-lipoic acid and EGF (materials normally produced in the body), than previously used nanoparticles. The EGF coating facilitates entry to cancerous cells,[13] via binding to the EGF receptor, and would be selective for cancerous lesions via the enhanced permeability retention effect.

## Methodology

### *Mouse EGF–α-Lipoic Acid Protein Complex Synthesis*

The mouse EGF (mEGF)–α-lipoic acid complex was synthesized using the carbodiimide reaction methodology used previously by us.[1] Briefly, the reaction is a 2-step process of linker activation followed by amide bond formation. The molar ratios of reactants are 120 α-lipoic acid:3200 sulfo-NHS (sulfo-N-hydroxysulfosuccinimide):1300 EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide):1 mEGF. The formation of the mEGF–α-lipoic acid complex is confirmed by the corresponding increase in mass of the product, measured using a hybrid dual-pressure linear ion-trap or orbitrap tandem mass spectrometer.

### *Nanoparticle Synthesis*

Forty-four nanometers of silver nanoparticles were prepared by the method of Lee and Meisel[23] as modified by Grabar et al.[24] Briefly, in a fume hood and under refluxing, 100 mL of 0.02% by weight silver nitrate solution (0.05 g silver nitrate and 250 mL deionized $H_2O$) was heated with stirring to boiling in a fume hood. At once, 2 mL of 1% sodium citrate was added to seed the formation of nanoparticles.

Forty-three nanometers of gold nanoparticles were prepared by the method of Grabar et al.[24] In a fume hood and under refluxing, 125 mL of a 0.04% by weight gold chlorate solution was stirred and heated to boiling. The flask was heated in a silicon oil bath to ensure even heat distribution. At once, 12.5 mL of 0.1% by weight sodium citrate was added to reduce the gold salt with continued boiling. The heating continued for 10-15 min until the color of the solution was stabilized.

### *Transmission Electron Microscopy*

Transmission electron microscope images were captured of samples blotted onto 300-mesh carbon-coated copper grids. A 20-μL drop of nanoparticles was spotted onto the grid and air dried overnight before imaging. The electron microscope (Tecnai 12, FEI, Hillsboro, OR) produced a beam of electrons at 80 keV, corresponding to a 0.0155-nm wavelength. Images were obtained using Digital-Micrograph™ software and a camera, both from Gatan (Pleasanton, CA). A minimum of 100 particles was counted with ImageJ software (NIH, Bethesda, MD) to obtain the particle size distribution, from which the mean particle diameter and standard deviation were derived.

### *Mass Spectroscopy*

A Thermo Scientific™ Orbitrap ™ mass analyzer and Velos Pro ion trap was used to deduce any change in mass of mEGF resulting from the reaction outlined previously. Five microliters of sample was diluted in 45 μL of 50% acetonitrile with 0.1% formic acid solution and loaded on coated nanoelectropray capillaries (GlassTip PicotTip Emitters, from NewObjective). Full-scan mass spectroscopy (MS) spectra were acquired using 1.5-kV ionization voltage on a Static Nanospray Ionization source (NS1) coupled to a Velos Pro-Orbitrap (Thermo Scientific) operated in linear ion trap and high resolution FT modes. Data were imported to Bioanalyst software for spectral deconvolution using Bayesian protein reconstruct algorithm.

### *Nanoprobe Synthesis and Characterization*

A comprehensive description of the synthesis and characterization of our nanoprobes has been reported elsewhere[1] wherein bioconjugation of EGF to silver nanoparticles was reported for the first time. Briefly, the modified mEGF has affinity toward the noble metals silver and gold on account of the α-lipoic acid modification. To ensure stability of the probes, the protein complex was added in excess to obtain complete coverage of the nanoparticle.[1] Please refer to the supplementary information for detailed synthesis procedure. In short, mEGF–α-lipoic acid solution was added to entirely cover silver nanoparticles (AgNPs) 42.5 nm, and gold nanoparticles (AuNPs) 44.1 nm. Nanoparticles coated solely with α-lipoic acid are made as controls for the mEGF-linker–covered nanoparticles.

The nanoparticle solutions remained under constant stirring for 24 h. After this time, they were purified using the method of Balasubramanian et al.,[25] to remove excess mEGF-linker and α-lipoic acid. The 42.5 nm of silver nanoparticles and 44.1 nm of gold nanoparticles were centrifuged at 10,000 rpm for 2 min to make a pellet, then resuspend in 1 mL of deionized water. This process was repeated once, and the resulting nanoprobe solution was sterilized using Sterile Filters (0.22-μm pore, 10-mm diam., GVWP01300—Millipore, Billerica, MA), inside a Swinnex Filter Holder (SX0001300—Millipore).

### *Animal Studies*

Male C57BL/6 mice were procured from Charles River (Wilmington, MA) at 8 weeks weighing 22-24 g, were housed in groups of 4 under standard environmental conditions (12-h light/dark photoperiod) and were maintained with free access to standard