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Visualization of molecular composition and functionality of cancer cells using nanoparticle-augmented ultrasound-guided photoacoustics

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ABSTRACT

Assessment of molecular signatures of tumors in addition to their anatomy and morphology is desired for effective diagnostic and therapeutic procedures. Development of *in vivo* imaging techniques that can identify and monitor molecular composition of tumors remains an important challenge in pre-clinical research and medical practice. Here we present a molecular photoacoustic imaging technique that can visualize the presence and activity of an important cancer biomarker – epidermal growth factor receptor (EGFR), utilizing the effect of plasmon resonance coupling between molecular targeted gold nanoparticles. Specifically, spectral analysis of photoacoustic images revealed profound changes in the optical absorption of systemically delivered EGFR-targeted gold nanospheres due to their molecular interactions with tumor cells overexpressing EGFR. In contrast, no changes in optical properties and, therefore, photoacoustic signal, were observed after systemic delivery of non-targeted gold nanoparticles to the tumors. The results indicate that multi-wavelength photoacoustic imaging augmented with molecularly targeted gold nanoparticles has the ability to monitor molecular specific interactions between nanoparticles and cell-surface receptors, allowing visualization of the presence and functional activity of tumor cells. Furthermore, the approach can be used for other cancer cell-surface receptors such as human epidermal growth factor receptor 2 (HER2). Therefore, ultrasound-guided molecular photoacoustic imaging can potentially aid in tumor diagnosis, selection of customized patient-specific treatment, and monitor the therapeutic progression and outcome *in vivo*.

1. Introduction

Molecular imaging techniques capable of good penetration depth in living tissue remain an important challenge in basic and clinical science including modern biology and medicine [1–4]. Optical imaging can provide unprecedented wealth of molecular specific information. However, tissue turbidity limits penetration depth of light *in vivo* to a few hundred micrometers for high-resolution imaging modalities such as confocal microscopy, optical coherence tomography (OCT), or two-photon fluorescence [5–8]. Approaches based on diffusely scattered light such as diffuse optical tomography (DOT) can extend this limit to several centimeters, but they suffer from low resolution and rely on complex reconstruction algorithms that require a priori knowledge of tissue optical properties. A unique solution to this problem is the recently emerging photoacoustic imaging technique that combines optical excitation and ultrasound detection [9–12]. This imaging approach relies on "one-way" propagation of diffusive photons into the tissue where the photoacoustic signal is generated through thermal interaction of pulsed laser light with photo-absorbers. Hence the contrast mechanism in photoacoustic imaging is primarily related to the optical absorption properties

Abbreviations: AuNPs, gold nanoparticles; EGFR, epidermal growth factor receptor; PA, photoacoustic.

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of the tissue being imaged. Beyond the depth of ballistic photons, the spatial resolution of photoacoustic imaging is determined by the ability of the ultrasound transducer to resolve the three-dimensional distribution of photoabsorbers that generate photoacoustic transient waves. Photoacoustic imaging can visualize optical absorption properties of tissue at sufficient depth, and salient features of photoacoustic imaging are described in several reviews in recent years [9–15]. Furthermore, a synergistic integration of photoacoustic imaging with clinically available ultrasound imaging systems is also possible and is being pursed [9–12].

Endogenous contrast in photoacoustic imaging is largely limited to hemoglobin and melanin molecules. In other applications, detection of lipid and collagen is possible with photoacoustic imaging [13,16–18]. Detection of other biomarkers or functionality associated with tumors requires availability of molecular probes or molecular specific contrast agents targeted to these biomarkers [14,15,19,20]. Plasmonic gold and silver nanoparticles are ideally suited for photoacoustics because of their high absorption cross-sections [10,11,20]. Molecular specificity is conferred to these plasmonic nanoparticles via conjugation to probe molecules such as antibodies [21,22]. However, the sole addition of a targeting moiety is often not sufficient for sensitive molecular imaging because of the background signal generated by the non-specific delivery of contrast agents to the imaging site. In cancer imaging, non-specific delivery of contrast agents is related to leaky vasculature of the tumor i.e., contrast agents accumulate in the tumor site primarily due to the enhanced permeability and retention (EPR) effect. As done in immunohistological protocols, extensive blocking and washing steps cannot be applied *in vivo* to remove non-specific binding. Multiple innovative strategies have been developed to enable highly specific molecular imaging – for example, in fluorescence imaging, various activatable probes and beacons are used to provide signal only in the presence of a biomarker of interest or detect a change in the signal on a cue from the tumor micro-environment [23–25]. However, again, most of these approaches are limited to optical modalities that do not possess sufficient penetration depth *in vivo*.

We and other groups have previously showed that targeted plasmonic nanoparticles by themselves can be used in a similar way as activatable contrast agents in molecular optical imaging [10,14,15,19,20,26–28]. The approach is based on the phenomenon of plasmon resonance coupling between closely spaced noble metal nanoparticles [26,27,29,30]. The coupling results in strong optical changes including red spectral shift and broadening of nanoparticle extinction spectra [21,26,27,29–31]. The formation of closely spaced assemblies can be mediated by specific interactions between targeted gold nanoparticles and a biomolecule of interest such as a cancer biomarker, e.g. epidermal growth factor receptor (EGFR) [31]. Confocal reflectance and dark-field optical imaging of EGFR positive cancer cells labeled with anti-EGFR antibody conjugated spherical gold nanoparticles showed a red shift of more than 100 nm in nanoparticle plasmon resonance frequency [27,31]. Further studies revealed that the observed optical changes are associated with EGFR activation and trafficking – key signaling pathways that determine cell behavior in normal and cancerous tissue [31]. As activated EGF receptors undergo dimerization and further aggregation in the plasma membrane, followed by internalization through endocytosis [31], the EGFR-targeted AuNPs associated with this process undergo a progressive change in optical properties (i.e., change in optical absorption) as schematically depicted in Fig. 1. Therefore, antibody targeted gold nanoparticles undergo dramatic optical changes upon binding to activated EGF receptors and endocytosis in live cells. We previously demonstrated molecular-specific photoacoustic imaging in three-dimensional cell culture phantoms and ex vivo tissue

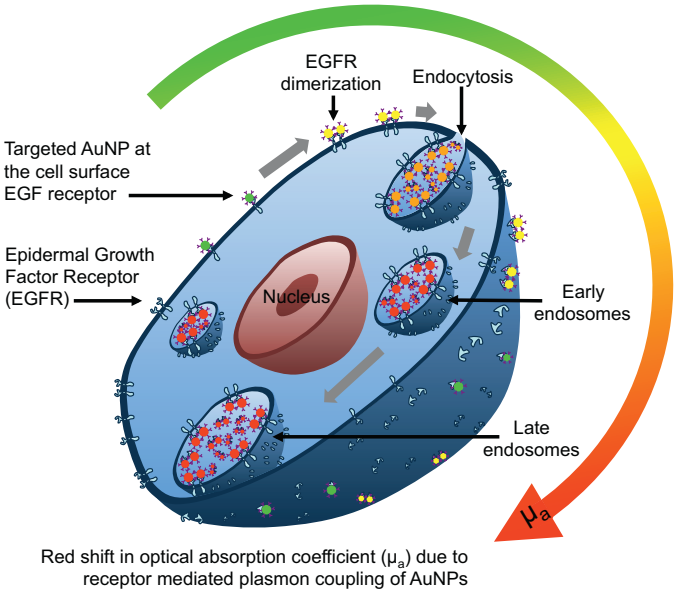

Fig. 1. Schematic showing change in optical absorption properties of EGFR-targeted AuNPs upon interaction with a cancer cell overexpressing EGFR. Activated EGF receptors undergo dimerization and further aggregation in the plasma membrane, followed by internalization through endocytosis. The EGFR-targeted AuNPs associated in this process undergo a progressive color change (i.e., change in optical absorption) from green to red and near-infrared as depicted in the absorbance spectra at various stages of AuNPs interaction with the cancer cell.

[21]. This approach is not unique to EGFR molecules and has also been applied to monitoring actin reorganization, detection of fibronectin–intergrin complexes, and imaging membrane morphology in live cells [30,31].

Here, we report multi-wavelength photoacoustic imaging of cancer cells in a xenograft murine tumor model *in vivo* using the effect of plasmon resonance coupling of EGFR-targeted gold nanoparticles. Specifically, when targeted AuNPs bind to EGFR molecules, trafficking of the labeled receptors results in receptor-mediated aggregation of AuNPs inside endosomal compartments causing plasmon resonance coupling between closely spaced AuNPs (Fig. 1). This leads to a strong increase in absorption (thereby increase in photoacoustic signal) in the red spectral region [26,27,29,31]. These changes in optical properties provide the unique opportunity for photoacoustic imaging to monitor molecular specific interactions between nanoparticles and cell-surface receptors, allowing visualization of the presence and functional activity of viable tumor cells.

2. Materials and methods

2.1. Photoacoustic imaging system

The combined ultrasound and photoacoustic imaging system (Fig. 2a) was based on an ultrasound engine (Winprobe Corporation, North Palm Beach, FL, USA) interfaced with either a Q-switched Nd:YAG laser (532 nm wavelength, 5 ns pulses, 20 Hz pulse repetition frequency) or a tunable OPO laser system (680–950 nm wavelength, 7 ns pulses, 10 Hz pulse repetition frequency). The laser fluences were within 10–20 J/cm^2 according to the American National Standards Institute (ANSI) safe exposure level for human skin. To image the tumor, an integrated imaging probe consisting of a 7.5 MHz center frequency ultrasound transducer (14 mm wide, and 128 element linear array) and a bundle of optical fibers for laser light delivery (Fig. 2b) was used. The axial, lateral,