Multiplex photoacoustic molecular imaging using targeted silica-coated gold nanorods

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Abstract: The establishment of multiplex photoacoustic molecular imaging to characterize heterogeneous tissues requires the use of a tunable, thermally stable contrast agent targeted to specific cell types. We have developed a multiplex photoacoustic imaging technique which uses targeted silica-coated gold nanorods to distinguish cell inclusions in vitro. This paper describes the use of tunable targeted silica–coated gold nanorods (SiO$_2$-AuNRs) as contrast agents for photoacoustic molecular imaging. SiO$_2$-AuNRs with peak absorption wavelengths of 780 nm and 830 nm were targeted to cells expressing different cell receptors. Cells were incubated with the targeted SiO$_2$-AuNRs, incorporated in a tissue phantom, and imaged using multiwavelength photoacoustic imaging. We used photoacoustic imaging and statistical correlation analysis to distinguish between the unique cell inclusions within the tissue phantom.

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imaging method, capable of imaging multiple distinct molecular signatures within a single image, is necessary for sensitive detection of molecular and cellular content of heterogeneous tissue that is typical in cancer. Molecular imaging methods previously developed, including optical tomography [1], micro-computed tomography (micro-CT) [2], nuclear imaging [3], and magnetic resonance imaging (MRI) [1], have limitations with respect to ease of use, safety, or cost. Ultrasound (US) imaging using contrast agents has been adapted for molecular imaging [4] due to the relative safety, high resolution, and affordability of the ultrasound technology. A complementary imaging mode, PA molecular imaging, has been demonstrated to be capable of imaging specific tumor cell types [5] with a sensitivity of 1.25 picomolar of targeted gold nanorod contrast agent [6].

Photoacoustic imaging uses pulsed laser light to generate ultrasound transients from optically absorbing materials through thermoelastic expansion [7]. Photoacoustic signals are recorded and used to construct an image of the optical properties of the tissue of interest, providing functional information about the tissue [8,9]. Photoacoustic signal can be enhanced using contrast agents, such as metallic nanoparticles which demonstrate surface plasmon resonance, providing high optical absorption of a tuned laser light followed by the generation of acoustic transients [10–12]. Gold nanoparticles have minimal toxicity and immunogenicity [13], encouraging the study of gold nanoparticles for biomedical imaging applications. In particular, gold nanorods are ideal PA contrast agents, because their optical absorption spectra can be tuned over a broad wavelength range in the near infrared (NIR) spectral region to take advantage of both the tissue optical window and the higher laser fluences allowable by ANSI standards, which are between 20 and 100 mJ/cm² in the NIR range [14]. Optical fluence decreases approximately 1 order of magnitude every 3 cm in breast tissue in the NIR [15], indicating laser fluences sufficient to generate PA signal from nanorod contrast agents can be achieved at several centimeters depth in clinically relevant tissues. However, these higher fluences will cause gold nanorods to change their optical absorption spectra due to the nanorods inherent thermal instability, which is accelerated upon exposure to pulsed laser light. Gold nanorods begin to demonstrate changes in optical absorption spectra at 8 mJ/cm² with as few as 300 laser pulses [16], suggesting that in vivo PA imaging within 1 cm of the skin surface would cause degradation, and therefore the loss of optimal optical properties, of the nanorod contrast agents. For these reasons, a silica-coating method of providing improved stability during PA imaging [16] has been used in this work.

The chosen targets of the multiplex nanoparticles, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), are both associated with poor patient prognosis in many cancers, including head and neck, ovarian, cervical, bladder and esophageal cancers [17–19]. Studying the combination of EGFR family of receptors can provide an improved prediction of patient prognosis [20]. In particular, HER2 is unique in that it is not activated by EGFR family ligands, only acting as a heterodimerization partner for the other EGFR family receptors. This feature makes HER2 a complementary choice for use as a indicator of prognosis in combination with EGFR [18]. Additionally, the cell lines chosen for this study, A431 and MCF7, have known cell receptor expression patterns: MCF7 cells present low expression of EGFR [21] and higher expression of HER2 [22], while A431 cells present high expression of EGFR [23] and low expression of HER2 [22].

The molecular photoacoustic imaging system demonstrated here improves upon previous in vitro multiplex photoacoustic imaging results [24,25] by using multiplex photothermally stable and tunable SiO₂-AuNRs which provide enhanced PA signal [16,26] and by acquiring multispectral PA data [27]. These improvements are necessary for successful adaptation of the multiplex imaging method to in vivo animal models where the spectra of the AuNRs must be explicitly resolved from endogenous contrast agents. Additionally, while SiO₂-AuNRs have increased stability as contrast agents for photoacoustic imaging [28,29], they also exhibit significantly enhanced PA signal in comparison to non-silica coated nanorods, acting as nanoamplifiers [26]. We also anticipate that the more rounded shape of the SiO₂-AuNRs will be advantageous to cellular uptake, since the high aspect ratio of nanorods decreases their endocytosis in cells in comparison to more spherical nanoparticles. In this work we have also
developed synthesis methods to directionally bioconjugate targeting molecules to the SiO$_2$-AuNRs, demonstrated their uptake in vitro, and the subsequent generation of PA images of the cellular targets. Using both multispectral PA imaging and targeted SiO$_2$-AuNR contrast agents, these methods distinguish between two different types of cells contained within inclusions in a tissue-mimicking phantom.

2. Materials and methods

2.1. SiO$_2$-AuNR Synthesis and Bioconjugation

The synthesis of silica-coated gold nanorods occurred through a multistep process as previously reported [16]. In summary, gold seeds were synthesized from gold(III)chloride hydrate in the presence of the surfactant cetyltrimethylammonium bromide (CTAB) by adding sodium borohydride. A nanorod growth solution was created by adding silver nitrate, gold(III)chloride hydrate, CTAB, and ascorbic acid to the seed solution [30]. The resulting nanorods were centrifuged at 18,000 g for 45 minutes and redispersed in ultrafiltered deionized water twice. Next, a layer of mPEG-thiol was formed on the surface of the gold nanorods through ligand exchange with the CTAB, followed by growth of an amorphous silica layer using tetraethyl orthosilicate (TEOS) via the Stöber method [29]. Transmission electron microscopy (TEM) images were acquired to characterize the composition and size of the resulting SiO$_2$-AuNRs.

Directional bioconjugation of the SiO$_2$-AuNRs was accomplished by functionalizing the silica-coated surface and using a heterofunctional crosslinker reported in previous procedures [31]. First, the silica coating of the nanorods was functionalized using (3-aminopropyl)tris(trimethylsiloxy)silane. Monoclonal antibodies targeted to HER2 and EGFR were conjugated to the amine functional group using the heterofunctional crosslinker sulfo-succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate [31]. Two distinct SiO$_2$-AuNRs were synthesized, the first with a peak optical absorption of 780 nm targeted to HER2, and the second with a peak optical absorption of 830 nm targeted to EGFR. The UV-Vis absorbance spectra of the two targeted SiO$_2$-AuNRs were measured using a microplate reader (Biotek Synergy HT).

2.2. Cell Culture

For these experiments, A431 cells, which over-express EGFR, and MCF7 cells, which over-express HER2, were cultured using standard techniques in Dulbecco’s modified eagle medium (DMEM) and incubated at 37°C with 5% CO$_2$ at 95% relative humidity. During typical cell culture, media was exchanged every two days and cells were passaged when 90% confluent.

2.3. In vitro SiO$_2$-AuNR uptake

To demonstrate effectiveness of targeting, cells were grown on glass coverslips until adherent, incubated with both targeted and non-targeted SiO$_2$-AuNRs dispersed in phenol red-free DMEM, for 24 hours, and then mounted on glass slides for optical microscopy. Brightfield microscopy images were acquired of the cells incubated with either the targeted or the non-targeted SiO$_2$-AuNRs, which had a bare silica surface. To quantify the uptake of gold nanorods, cells incubated with either the targeted or the non-targeted SiO$_2$-AuNRs were harvested, acid digested and the quantity of gold in each sample was analyzed by inductively coupled plasma mass spectrometry (ICP-MS). To calculate the number of SiO$_2$-AuNRs nanoparticles per cell, the amount of gold per nanoparticle was calculated based on the quantity of gold added during synthesis of the SiO$_2$-AuNRs and the volume of gold contained within each SiO$_2$-AuNR as estimated from TEM images. The cell concentration of the cell solution harvested after incubation with the SiO$_2$-AuNRs was calculated using a hemocytometer, so that the quantity of gold detected by ICP-MS can then be used to calculate the number of nanoparticles per cell.
2.4. In vitro photoacoustic imaging

To demonstrate multiplex photoacoustic molecular imaging in vitro, a tissue-mimicking phantom containing inclusions of both A431 and MCF7 cells loaded with the specifically targeted \( \text{SiO}_2\)-AuNRs was created. Cultures of adhered cells were incubated with \( 1 \times 10^{12} \) NRs/mL for 24 hours. Cells were then harvested and resuspended in phenol red-free media at a concentration of \( 1 \times 10^6 \) cells/mL. The tissue phantom (8% w/v gelatin, 1.2% w/v 5 \( \mu \)m diameter silica scatterers) was constructed to contain 20 \( \mu \)L inclusions consisting of the gelatin solution mixed 1:1 with the cell samples.

As shown in Fig. 1, a tunable OPO system pumped by a pulsed Nd:YAG laser was used to irradiate and generate PA signal from the tissue phantom at several wavelengths between 700 nm and 910 nm. A transducer (25 MHz, 60% fractional bandwidth, f/#4, 25 mm focal depth) was used to collect both US and PA signals. The transducer was moved using a 1D axis in 100 \( \mu \)m steps, and radiofrequency (RF) signals were acquired at each step. The ten RF-signals acquired at each lateral position were averaged before to produce US and PA images [32].

![Fig. 1. Custom-built system used to acquire combined US and PA images of cell phantom.](image)

Correlation between the wavelength-dependent PA signal intensity and the UV-Vis optical absorption spectra was performed using an intraclass correlation (ICC) [32]. A correlation method, in contrast to a regression analysis, makes no assumptions about the variable dependence. The ICC was chosen as an assessment of the agreement between different methods used on the same set of subjects. In comparison to other common correlation methods, such as Pearson’s correlation, the ICC does not require the assignment of the two measurement methods to a particular order. A threshold of 0.75 was used to identify pixels which showed high correlation (\( \geq 0.75 \)) or did not correlate (<0.75) to the nanoparticle UV-Vis absorbance spectra.

3. Results and discussion

As shown in Fig. 2a and Fig. 2b, the synthesized gold nanorods are uniformly coated with silica with an approximate thickness of 40 nm. The UV-Vis spectra of the two targeted \( \text{SiO}_2\)-AuNRs are shown in Fig. 2c and Fig. 2d. The maximum peak absorption wavelengths were 780 nm and 830 nm. The absorption of the gold nanorods in the NIR is intended to improve the depth at which the nanorods could be imaged in future in vivo experiments.

Targeting of the \( \text{SiO}_2\)-AuNRs using monoclonal antibodies specific for over-expressed cell receptors results in an increase in the cellular uptake. Brightfield microscopy (Fig. 3a and Fig. 3d) shows an increased amount of \( \text{SiO}_2\)-AuNRs uptaken by cells incubated with the targeted \( \text{SiO}_2\)-AuNRs, in comparison to cells incubated with the non-targeted \( \text{SiO}_2\)-AuNRs (Fig. 3b and Fig. 3d), indicated by the areas which appear pink within the cells. The increased uptake of the targeted \( \text{SiO}_2\)-AuNRs was quantitatively confirmed by ICP-MS. The number of
Fig. 2. Characterization of multiplex SiO$_2$-AuNRs. TEM images showing the size of the as-synthesized SiO$_2$-AuNRs (prior to bioconjugation) with peak optical absorbances of 780 nm (a) and 830 nm (b). UV-Vis spectra showing the peak optical absorption of the targeted SiO$_2$-AuNRs are shown in (c) and (d).

| Targeted Si-NRs | Non-targeted Si-NRs | Cells Only |
|----------------|---------------------|------------|
| A431 Cells     |                     |            |
| a)             | b)                  | c)         |
| MCF7 Cells     |                     |            |
| d)             | e)                  | f)         |

Fig. 3. Optical microscopy images demonstrating enhanced uptake of targeted silica-coated gold nanorods (SiO$_2$-AuNRs). a) SiO$_2$-AuNRs targeted to the EGFR receptor are uptaken in greater amounts in A431 cells in comparison to b) non-targeted SiO$_2$-AuNRs with an identical aspect ratio. Likewise, d) SiO$_2$-AuNRs targeted to the EGFR show increased uptake in A431 cells in comparison to e) non-targeted SiO$_2$-AuNRs. Cells which have not been exposed to SiO$_2$-AuNRs are shown in panel c) and panel f) as controls. Images obtained using a 20 × objective (0.5 NA) and Leica 6000 DM microscope.

SiO$_2$-AuNRs nanoparticles per cell was calculated to be $3 \times 10^5$ when HER2 targeted SiO$_2$-AuNRs were incubated with MCF7 cells (2x greater than untargeted SiO$_2$-AuNRs), and $5 \times 10^5$ when EGFR targeted SiO$_2$-AuNRs were incubated with A431 cells (13x greater than...
untargeted SiO\textsubscript{2}-AuNRs). Since MCF7 cells express approximately $1 \times 10^4$ HER2 receptors/cell [33], while A431 cells express approximately $2 \times 10^6$ EGFR receptors/cell [34], the increase in the uptake of gold in the A431 cells in comparison to the MCF7 cells seen in the ICP-MS results would be expected. The calculated number of nanoparticles per cell is similar to other results quantifying the cellular uptake of nanoparticles in vitro [32,35]. In vivo, directional bioconjugation of cell receptor targeting antibodies to the SiO\textsubscript{2}-AuNRs will increase the amount of nanoparticles uptaken and retained within a cancerous tumor [36], increasing the sensitivity of the PA imaging method to the molecular heterogeneity of tissue.

An US image of the tissue phantom shows the presence of inclusions, but the inclusions containing SiO\textsubscript{2}-AuNRs cannot be distinguished from inclusions which do not contain SiO\textsubscript{2}-AuNRs (Fig. 4a). Strong PA signal intensities identify the presence of nanorods in the expected inclusions, while the control inclusions that have no photoabsorbers exhibit no PA signal (Fig. 4b). However, since nanorods of differing optical absorption spectra – the multiplex SiO\textsubscript{2}-AuNRs - were used to target different cell types, the cell types can be identified using the multispectral PA imaging signals. An agreement between the UV-Vis spectra of the SiO\textsubscript{2}-AuNRs and the wavelength-dependent PA signal intensity of each cell inclusion can be seen (Fig. 4c). An intraclass correlation (ICC) between the multi-wavelength PA signal and the optical absorption spectra of SiO\textsubscript{2}-AuNRs measured by UV-Vis allows for the distinction between the two different cell types within the tissue phantom (Fig. 4d). In this image, ICC values greater than 0.75 correlating the PA signal intensity and the UV-Vis spectra of the $\lambda_{\text{max}} \approx 830$ nm SiO\textsubscript{2}-AuNRs are plotted in red, while ICC values greater than 0.75 correlating the PA signal intensity and the UV-Vis spectra of the $\lambda_{\text{max}} = 780$ nm SiO\textsubscript{2}-AuNRs are plotted in yellow, clearly defining the locations and boundaries of the cell inclusions. In this case, we have used SiO\textsubscript{2}-AuNRs with peak optical absorption wavelengths separated only by 50 nm. However, due to the width of the optical absorption peak, we were unable to identify the presence of both nanorods mixed within a single inclusion. Improvements in the synthesis of homogeneous nanorods with very narrow UV-Vis absorption bands will permit the distinction between nanorods which are less than 50 nm apart in their peak optical absorption wavelength.

![Fig. 4. Signal processing and statistical analysis of the PA images acquired from the cell phantoms demonstrates the unique identification of the cell inclusions. a) The inclusions can be seen in the ultrasound image. b) The PA image, acquired at 830 nm, indicates which inclusions contain SiO\textsubscript{2}-AuNRs. c) Comparison of PA signal intensity (points) and UV-VIS spectra (solid lines) demonstrates that the SiO\textsubscript{2}-AuNRs optical absorption spectra determine the PA signal intensity. Inclusions were segmented into three areas and the PA signal intensity was averaged; error bars represent one standard deviation (n = 3). d) Molecular map of cells and US overlay; 830 nm SiO\textsubscript{2}-AuNRs are shown in red, 780 nm SiO\textsubscript{2}-AuNRs are shown in yellow (ICC > 0.75), FOV = 3.5 mm x 53 mm, US image dynamic range = 35 dB, PA image dynamic range = 10 dB.](image)

4. Conclusions

Using photothermally stable SiO\textsubscript{2}-AuNR, which have enhanced PA signal in comparison to PEGylated AuNRs, and a multispectral PA imaging methodology, our studies demonstrate an...
approach which can be used to identify multiple cell types within heterogeneous tissue. By analyzing the PA signal intensity as a function of laser wavelength, we correlate the PA signal to the optical absorption spectra of the SiO$_2$-AuNR contrast agents. Since two different SiO$_2$-AuNR contrast agents were used to label each unique cell type, we can identify the location of the specific cell types and generate a molecular image of the tissue phantom. These improved multiplex PA imaging methods, which are demonstrated in vitro in this work, will enable the implementation of multiplex PA imaging in vivo.

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