Gold Nanorods as Contrast Agent for Photoacoustic Imaging (PAI) of Breast Cancer

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Abstract. Gold Nanorods (AuNRs) can play a fundamental role in the enhancement of photoacoustic imaging (PAI). This is due to their high and tunable optical absorption cross-section. Normally, the most convenient and highly stable AuNRs are synthesized using Cetyltrimethylammonium bromide (CTAB) as a surfactant. In this work, surface modification with poly ethylene glycol (PEGylation) and Silica coating ($\text{SiO}_2$-coating) were applied to help in reducing toxicity of CTAB capped AuNRs. Breast cancer cell line (MDA-MB-231) was used to assess the modified AuNRs as contrast agents. A much brighter PAI of cells loaded with $\text{SiO}_2$-coated AuNRs was obtained. Such an enhanced PA image is due to the larger cellular uptake, as observed from bright field microscope images taken for the cells. On the other hand, the corresponding PA image for cells with PEGylated AuNRs is diminished. The increase in cell uptake, in case of $\text{SiO}_2$-coated AuNRs, could result due to the adsorption of protein, contained in the cell culture media, on the negatively charged silica surface, which increases intercellular uptake by receptor-mediated endocytosis. Moreover, the increase of the brightness of PAI of $\text{SiO}_2$-coated AuNRs may be due to the lowering of the interfacial thermal resistance between gold and the surrounding solvent. This allows the particle to release more heat to its environment which increases the generated acoustic signals from these particles.

Keywords: Photoacoustic Imaging, contrast agents, Gold nanorods, Cancerous cells.

1. Introduction

Photoacoustic imaging (PAI) is a biomedical imaging technique based on the photoacoustic effect discovered by A.G. Bell in 1882(1). The photoacoustic effect refers to the conversion of light energy into acoustic energy. In PAI, a non-ionizing laser pulses are delivered into the biological tissue, some of the delivered energy will be absorbed and then converted into heat energy. This leads to transient thermoelastic expansion and thus wide band ultrasonic emission. The generated waves are detected by ultrasound transducer and then analyzed to produce images. The motivation for PAI is to combine ultrasonic resolution with high contrast due to light absorption. Thus, in the PAI ultrasound is detected and not light. The ultrasound energy experience less scattering and attenuation, therefore, a good spatial distribution of optical absorption can be obtained in deep tissues with high resolution and high penetration depth compared to other optical imaging modalities. Photoacoustic imaging and microscopic applications shows a great potential in ex-vivo, in-vivo, and in-vitro settings, due to its advantageous technique compared to other imaging techniques. The optical absorption is the primary contrast mechanism in photoacoustics, as it possesses information regarding the presence of tissue components such as hemoglobin, and melanin. Those components are considered as endogenous contrast agents. Strong optical absorption by hemoglobin and oxy-hemoglobin allows the visualization of blood vessels. Furthermore, those
contrast agents have a specific absorption spectrum which makes them distinguishable from other tissue components. Their presence gives more pathological and functional information about the imaged tissue. On the other hand, many exogenous contrast agents were used to enhance the photoacoustic signal. Gold nanoparticles are very interesting PA contrast agents (2-4). This is because they have a large absorption cross-section, due to the surface plasmon resonance (SPR) effect. Moreover, they have a high tunability as the absorption band position can be controlled by changing the nanoparticle size and shape. AuNRs are more preferred, because they have strong longitudinal SPR band lying in the optical biological window (700-1000nm), where the absorption of light by tissue is minimal. This condition is important for PAI of deep tissues that cannot be obtained by many other imaging methods. Therefore, AuNRs (5) have been developed and applied in both in-vitro and in-vivo applications. They are also functionalized for further enhancement in PAI contrast (6-10). AuNRs aspect ratios (length/width) between 3 and 4 are favored for PAI because their longitudinal SPR band lies at the biological window (11-19).

Silica (SiO$_2$) coating is considered as one of the most chosen functionalization methods for AuNRs. It is applied in many applications like PAI, cell-tracking, targeted drug delivery, and Surface Enhanced Raman Spectroscopy. This is due to their high stability and biocompatibility and functionality, compared to conventional CTAB capped AuNRs (20-22). In case of SiO$_2$-coated rods, the optical properties does not change in such techniques using a highly powerful laser, while in the case of CTAB capped AuNRs, melting takes place and there will be a shift in the longitudinal surface plasmon resonance band towards visible (23). Moreover, SiO$_2$-coating prevents particle aggregation and plasmon coupling effect at high particles concentration. Also, the surface area of particles increases due to silica coating, this help in more conjugation of antibodies or any other moieties.

In this work, three gold nanorods (AuNRs) samples were prepared via the seed mediated growth technique(12), where silver nitrate (AgNO$_3$) with different amounts were added to change the rods size. The PA image for particles in gelatin phantom is taken at different wavelengths using optical parametric oscillator (OPO) Laser, to obtain the wavelength for the maximum acoustic signal, for each particle size in gelatin inclusions. PA images are taken for MDA-MB 231 breast cancer cells loaded with PEGylated AuNRs and another cell loaded with SiO$_2$-coated AuNRs to assess the enhancement of PA image in each case.

2. Experiment

2.1. Synthesis and characterization of AuNRs with different sizes

The AuNRs were prepared using the seed-mediated growth technique (12). The seed solution was obtained by mixing 5mL of 0.2 M (CTAB, Sigma) with 5 mL of 0.5 mM HAuCl$_4$, and 0.6 mL of ice-cold 10 mM sodium borohydride (NaBH$_4$, Sigma), was then added. This results in the formation of a brownish yellow solution. Vigorous stirring of the seed solution was continued for 2 minutes. Afterword’s, the seeds were left for two hours before adding to the growth solution. The growth solution was obtained by mixing (900 mL, 0.1 M) CTAB with (50 mL, 10 mM) HAuCl$_4$, and (15-20-25) ml of 4 mM AgNO$_3$ (Sigma) then (10 mL, 0.1M) ascorbic acid was added drop wise. The solution then becomes colorless. Finally, 10 mL of the seed solution was then added to the growth solution. The color of the solution gradually changed within 20 minutes. The solution is set for overnight before concentration. We thus obtained three AuNRs solution with different longitudinal plasmon resonance peaks. The AuNRs were centrifuged twice at 11,000 rpm for 10 minutes and then re-dispersed in deionized water to remove the excess CTAB molecules.

CTAB, on the surface of the AuNRs was replaced by methoxy thiolated polyethylene glycol (mPEG-SH, MW 5000, Laysab Bio.) through ligand exchange. The CTAB-stabilized AuNR dispersion was added to an equal volume of (0.2 mM) mPEG-thiol aqueous solution. The solution was shaken at lowest speed overnight allowing CTAB ligands to exchange with mPEG-SH. The excess mPEG-SH was removed via centrifugation at 10,000 rpm for 10 minutes for two times. And the PEGylated AuNRs were re-suspended in water.

SiO$_2$-coating is carried out using the method in (21). In brief, Tetraethyl orthosilicate (TEOS) is used to get a mesoporous siloxane polymer on the surface of AuNRs. About 1.2 mL of PEGylated AuNR colloidal solution was mixed with 1.8 mL of isopropanol (IPA) under vigorous stirring, then an ammonia solution in IPA (3.84 v% ammonia (33 wt%)) was added slowly under vigorous stirring until the solution reached PH=11. Finally, (0.40
mL, 100 mM) solution of TEOS in IPA was added under gentle stirring. The reaction mixture was allowed to react for 2 hours.

The samples were characterized by UV–Vis spectroscopy (Ocean Optics), to determine their optical absorption spectra. Zetapotential (Zeta sizer Nano Series, Malvern) was measured to give an indication to the surface charge of the different particles. The particle size was determined using transmission electron microscopy (TEM). The samples were inspected using a JEOL 100 CX-II TEM operating at 100 kV. The TEM imaging of gold nanoparticles were carried out by placing the nanoparticles on carbon-coated copper grid and dried overnight in air. All of the micrographs were recorded on a CCD camera, and the images were analyzed using Image J software to get the particle size and the standard deviation.

2.2. Cell culture

MDA-MB-231 Breast cancer cell line is used to study the effect of PEGylation and Silica coating of gold nanorods as contrast agent for PA imaging. The used cell culture media consists of Dulbecco's modified eagle medium: Nutrient Mixture (DMEM/F12), 10% fetal bovine serum, and 2% penicillin/streptomycin sulphate. A 37°C with 5% CO2 incubator was used for cell incubation. Cell media were changed every three days. 1mL of trypsin is then added to remove the cells when about 90% confluent is reached. Trypsin is an enzyme that breaks down proteins which enables the cells to adhere to the cell culture plate. Cells are then counted using a hemocytometer and reseeded in a 6-well cell culture plates, having about 5x10^5 cell in each well, and then let to adhere overnight before nanoparticles addition.

To incubate the particles with the MDA-MB-231 cancer cells, concentrated PEGylated and SiO\textsubscript{2}-coated rods are resuspended into phenol red-free DMEM/F12 cell media to known optical density (OD), the standard DMEM/F12 solution was aspirated from the cultured cells, and replaced with equal volume of nanoparticles in phenol red free DMEM/F12, such that the OD of the solution is 1. The cells are placed in the incubator for 24 hours at 37 °C. After incubation, excess nanoparticles were removed by gently aspirating and dispensing phosphate-buffered saline (PBS) for 3 times. Cells are then passaged with trypsin and the trypsin is quenched with cell media. The cells are then centrifuged and fixed with 10% formalin, centrifuged, and resuspended in cold PBS. A Tissue mimicking phantom with loaded cell inclusions was prepared for photoacoustic imaging. The cell suspension (20 μL) was mixed with gelatin solution (6% by weight after mixing) and placed on the base layer of the gelatin phantom.

2.3. Combined US-PA imaging

Combined US-PA images for the gelatin tissue-mimicking phantoms were obtained using Vevo 2100 ultrasound micro imaging system (VisualSonics, Inc.) described in (24). PA images for the different three AuNRs inclusions are take over the whole range of the laser source (680-930 nm). The PA spectra as a function of wavelength is obtained from the corresponding PA images by subtracting the average PA signal, calculated over the total area of AuNRs inclusion, from the background signal having the same area. PA images were taken for two gelatin cell inclusions, one having cells loaded with PEGylated AuNRs and the other one contains cells loaded with SiO\textsubscript{2}-coated AuNRs.

3. Results and Discussion

The UV-Vis absorption spectra for the three prepared samples with different amounts of AgNO\textsubscript{3} (15ml, 20ml, 25 ml) as mentioned in the experimental part, are shown in Figure 1(a). The obtained longitudinal SPR peaks were at 670 nm, 767 nm, and 838 nm, respectively, while the transverse mode is at 520 nm for all samples. Figure 1(b) shows the UV-Vis spectra of PEGylated and SiO\textsubscript{2}-coated AuNRs of sample (2) in Figure 1(a). There is about 20 nm red shift to the longitudinal surface plasmon resonance peak between the PEGylated and SiO\textsubscript{2}-coated AuNRs, which confirms the coating process.
Figure 1 (a) UV-Vis spectra for AuNRs with different added amounts of AgNO3 ((1):15ml, (2):20ml, and (3):25ml), (b) UV-Vis absorption of blue: PEGylated and red: silica coated gold nanorods.

Figure 2(a), (b) shows the TEM images taken for the samples (1) and (2) in Figure 1(a), respectively. Figure 2(a) shows monodispersed nanorods with length = 35.93 ± 5.2 nm, width = 8.94 ± 1.08 nm, and aspect ratio of about 3.72. Figure 2(b) gives monodispersed nanorods with length = 56.71 ± 5.76 nm, width = 15.25 ± 2.32 nm, and aspect ratio of 4.02. The TEM image of the later sample coated with SiO2 of 40 nm thickness is shown in Figure 2(c).

Figure 2 TEM images of AuNRs with (a) length = 35.93 ± 5.2 nm, width = 8.94 ± 1.08 nm, (b) length = 56.71 ± 5.76 nm, width = 15.25 ± 2.32 nm, (c) sample b with 40 nm silica shell.
Zeta potential measurements were carried out for both PEGylated and SiO$_2$-coated AuNRs to confirm the coating process. 

Figure 3 shows that there is a change in the Zeta potential from +5.46 mV, for PEGylated AuNRs, to -45.8 mV due to the silica coating of AuNRs. The high negativity of SiO$_2$-coated AuNRs results in an increase of its surface free energy. This leads to better interaction of nanoparticles with proteins to reduce its surface free energy.

Figure 3 Zeta potential of (a) PEGylated and (b) Silica coated rods respectively

Figure 4 shows the PA spectra for the prepared AuNRs. Gelatin inclusions for the three prepared AuNRs samples having different aspect ratios are prepared at the same OD (3). The photoacoustic spectra from the tissue-mimicking phantom were obtained using the combined US-PA imaging system with OPO as light source. Figure 4 also shows the corresponding US-PA overlay image for the AuNRs with three different sizes, using the laser wavelength that gives maximum acoustic signal in each case. It is clear that the PA signal is maximum at LSPR wavelength obtained by UV-Vis spectra (Figure 1).

Figure 4 PA spectra for gelatin inclusions with AuNRs, with different aspect ratios shown in Figure 1a (1,2,3). Overlay US-PA images for gelatin inclusions taken at the shown corresponding peak wavelength are on top

Figure 5 shows the PA spectra for gelatin inclusions of MDA-MB-231 cells loaded with (a) PEGylated, and (b) SiO$_2$-coated AuNRs, as a function of wavelength (680 nm – 930 nm). It is found that the PA signal is maximized at about 805 nm, in case of cell loaded with SiO$_2$-coated AuNRs, while in case of PEGylated nanorods there is no signal over the whole range. An overlay US-PA images for cells loaded with PEGylated (a) and SiO$_2$-coated (b) AuNRs are shown in Figure 6. These images were taken at laser excitation which gives maximum PA signal.
The diminished PA signal for cells loaded with PEGylated AuNRs is due to the reduced cellular uptake of PEGylated AuNRs compared to SiO\textsubscript{2}-coated AuNRs, as indicated from the bright field microscope images.

![Graph showing PA signal at different applied laser wavelengths for cells loaded with (a) PEGylated, and (b) silica coated gold nanorods](image)

**Figure 5** PA signal at different applied laser wavelengths for cells loaded with (a) PEGylated, and (b) silica coated gold nanorods

![Overlay US-PA images for cells loaded with (a) PEGylated and (b) silica coated AuNRs](image)

**Figure 6** Overlay US-PA images for cells loaded with (a) PEGylated and (b) silica coated AuNRs

Figure 7 shows the bright field images obtained using 20x objective (0.5 NA) and Leica 6000 DM microscope for the loaded cells. The images confirm that the cell uptake for SiO\textsubscript{2}-coated AuNRs is more than that of PEGylated rods. It is evident that the silica coating enhances the cell uptake as well as the PA signal. This result agrees with the study of Xiao-Ming Zhu (25).
The enhanced cellular uptake in case of SiO$_2$ coated AuNRs, is due to the net negative charge on the nanoparticle surface (as found in the Zeta potential measurements). This negative charge produces electrostatic interaction with positively charged function groups in proteins contained in the cell culture media via fetal bovine serum. Therefore, the protein corona that surrounds the SiO$_2$-coated AuNRs, induce the rods to enter the cell via receptor-mediated endocytosis. Wang L M et al (26) found that bovine serum albumin, in cell media, adsorb on surface of gold nanoparticles by about twelve Au-S bonds. On the other hand, protein complexes cannot be formed in case of PEGylated gold nanoparticles. Since the Zeta potential is almost neutral, therefore, they have a high capacity to resist the adsorption of proteins after modification with high densities of hydrophilic PEG (27) and hence, circumvent cellular uptake.

Further enhancement in the PA signal, for cells loaded with SiO$_2$-coated AuNRs, can be attributed to the assistance of silica shell in reduction of the interfacial thermal impedance between gold and surrounding solvent which allows particles to release more heat, leading to larger acoustic signal (28).

4. Conclusions

Though PEGylation and Silica coating help in reducing toxicity of CTAB capped AuNRs, however PEGylation diminish the PAI of AuNRs loaded cancer cells, due to reduction of cellular uptake of PEGylated AuNRs. As a result of incubating SiO$_2$-coated AuNRs with cancer cells, an enhanced cellular uptake is observed for silica coated AuNRs compared to the diminished PEGylated NRs. this enhanced PA image is due to the formation of a protein corona around SiO$_2$-coated AuNRs which increase intercellular uptake by receptor-mediated endocytosis. Moreover, the increase of the SiO$_2$-coated AuNRs is also because the silica coating helps lowering the interfacial thermal resistance between gold and the surrounding solvent which allows the particle to release more heat to its environment. This increases the generated acoustic signals from these particles.

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