Size effect of gold nanospheres on the photoacoustic imaging of cancerous cells

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Abstract. The size effect of gold nanospheres (AuNS) used as a contrast agent for the photoacoustic imaging (PAI) of breast cancer cell line MDA-MB-231 have been assessed for three different sizes and at four optical densities (ODs). The decrease in the AuNS size at the same OD results in an improved PA signal. The PA signal generally increases linearly with increasing OD, with almost the same rate of increase for the three used particle sizes. It is shown that PEGylation of gold nanoparticles (AuNP) decreases the uptake of the particles by the cancerous cell therefore diminishes the PA images whereas clear images are obtained in the case of unPEGylated AuNPs.

Keywords: Photoacoustic Imaging, Gold nanoparticles, Cancerous cells.

1. Introduction

Photoacoustic imaging (PAI) has recently become of great relevance in biomedical imaging[1-4], and has captured the attention of biomedical engineers, applied physicists, applied mathematicians, and clinical imaging specialists[5]. The technique assembles the biomedical optics and ultrasound which leads to great improvement in imaging [6-8]. The PA technique is based on the photoacoustic effect that was first observed by Alexander Graham Bell in 1880[9]. It uses short- pulsed (or modulated) electromagnetic radiation as probing energy, while detecting ultrasound wave generated due to photon absorption and thermal expansion. There are a lot of advantages of PAI technique over many other imaging techniques, for example it has higher spatial resolution than that of fluorescence and a larger imaging depth, due to the much weaker scattering of ultrasonic signals than light in tissues[10]. Also, the PAI has better tissue contrast than that of ultrasound imaging, since the contrast of the latter is limited by the mechanical properties of biological tissues[11]. Moreover, the absence of ionizing radiation makes this technique safer than Computed Topography and radio nuclide-based imaging techniques[12]. In addition, measuring PA signal at different modulation frequencies one can retrieve individual contribution of scattering and absorption by applying Kubelka –Munk theory [13-16].

Gold nanoparticles (AuNPs) are considered one of the most promising exogenous PA contrast agents [17-20]. This is due to their strong and tunable optical absorption that results from the surface plasmon resonance (SPR) effect. SPR occurs when free charges on the surface of AuNPs oscillate with the electromagnetic field, leading to an optical absorption. Such absorption is several orders of magnitude greater than that of organic dyes[21]. Furthermore, the size and shape of the AuNP determine its resonant frequency, and absorption cross section which allows for fine tunability[22-24]. In this study, we have carried out an investigation of the effect of the size of AuNPs as a contrast agent on the PA imaging. The used particles were gold nanospheres(AuNS) of three different sizes ~12 nm, 66 nm, and 90 nm, that were synthesized by seed-mediated growth technique (as the larger sizes...
produced by citrate reduction were not stable or spherical). The AuNS were characterized by UV-Vis absorption, transmission electron microscope (TEM), Dynamic light scattering (DLS), and Zeta potential measurements. The AuNPs are then applied to breast cancerous cells of type MDA-MB-231 at different optical densities (ODs) to assess the effect of AuNP size on the PA signal.

2. Experimental Setup

2.1. Synthesis and characterization of gold nanospheres with different sizes

AuNS were synthesized using the seed-mediated growth technique as described in[25]. A 100 ml volume of 0.27 mM HAuCl4 in deionized water was brought to a boil. A 5.28 ml volume of 34 mM sodium citrate was added to the solution under vigorous stirring and allowed to stirr for several minutes. Then the solution temperature was allowed to return to room temperature. The synthesized nanospheres were of size ~ 12 nm. Larger nanospheres were synthesized using 7.5 ml of 25 mM HAuCl4 mixed with 15.61 ml of 0.2 M hydroxylamine hydrochloride NH2OH.HCl and 750 ml of deionized water. A 50ml and 12.5 ml of the as-prepared seed solution were added to the solution under vigorous stirring. After nanoparticles were allowed to sit overnight, they were characterized by UV–Vis spectroscopy (Ocean Optics), to determine their optical absorption properties. Zetapotential (Zeta sizer Nano Series, Malvern) was used to determine the surface charge. The particle size was determined using dynamic light scattering DLS, and transmission electron microscope TEM (JEOL 100 CX-II).

A solution of 1mg/ml methoxy polyethylene glycol thiol (mPEG-SH) of 5K molecular weight was added to same volume of a solution of citrate-capped Au nanoparticles in order to increase the particle stability and biocompatibility[26]. The solution was shaken at lowest speed overnight allowing citrate ligands to exchange with mPEG-SH. The excess mPEG-SH was removed via centrifugation at 7000 rpm for 10 minutes. The resulting colloidal solutions were stable (for several months) as there is no change in the UV-Vis absorption spectrum measured after 3 months.

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. Hydrodynamic size for the prepared particle was measured by Malverm Zeta sizer using the default non-invasive back scattering (NIBS) technique at 25 °C. The scattered light is measured using a detector at an angle of 173° for the particles sizes used (according to the instructions).

2.2. Cell culture

Breast cancer cell line, MDA-MB-231, used in this work to assess the effect of the size of gold nanospheres as a contrast agent on PA imaging, were cultured in Dulbecco’s modified eagle medium: Nutrient Mixture (DMEM/F12) supplemented with 10% fetal bovine serum, 2% pencilline/streptomycin sulphate and incubated at 37 °C with 5% CO2 at 95% relative humidity. Media were exchanged every 2–3 days and the cells were passaged with trypsin when 90% confluent. Cells are then counted using a hemocytometer and reseeded in 6-well cell culture plates of about 5x10⁵ cell per well, and then allowed to adhere overnight prior to the addition of nanoparticles. For each of the three different sizes, four different OD’s of 0.5, 1, 3, and 4, were prepared in phenol red-free DMEM/F12 cell media Optical Density (the optical attenuation per cm of solution OD). The particles were then incubated with the cells for 22 hours. After incubation; the media was aspirated, cells were then washed with phosphate buffered saline (PBS) 3 times to get rid of any unattached particles to the cells. In order to fix the cells in the plates; 2ml of 10% formalin were added for 10 minutes. The loaded cells are then covered with PBS and kept in the fridge for the experiment. For combined Ultrasound (US)-PA imaging, the loaded cells passaged with trypsin and the trypsin is then quenched with media. The cells are then centrifuged and fixed with 10% formaline, 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. PA measurements

The PA images for the tissue-mimicking phantom were obtained using a combined ultrasound (US)-PA transducer. US and PA signals were captured using a 40 MHz array transducer with a Vevo 2100 ultrasound micro imaging system (VisualSonics, Inc.) as is schematically presented in Figure 1a. A tunable OPO laser beam (Premiscan, GWU, Inc.) pumped by a pulsed Nd:YAG laser (Quanta-Ray, Spectra Physics, Inc.) was delivered through an optical fiber bundle (Ceramoptec, Inc.). The fiber bundle was combined with the ultrasound transducer and aligned such that the transmitted light would focus at the focal point of the array transducer. The trigger signal from the laser system was synchronized with the Vevo 2100 imaging system to capture the photoacoustic signal when the pulsed laser irradiated. In general, combined US and PA images when overlayed would indicate the locations of aggregated AUNS on the malignant cells on the background of the US topographic image. The experimental set up used for measuring only the PA signal is shown in Figure 1b. In this experiment, the excitation is generated by a Q-switched, pulsed Nd: YAG laser (Quanta-Ray PRO-290, Spectra-Physics Lasers, Mountain View, CA, USA). The laser output was at a wavelength of 532 nm, with a pulse-width of 5-7 ns and a pulse repetition rate of 10 Hz. The laser beam is split to two beams, the first beam is measured with a powermeter, and the second beam is focused on the sample using a plano-convex lens. PBS is used as an acoustic coupling medium to reduce the acoustic impedance between the sample and the ultrasound receiver. The induced PA pressure wave is measured by a 1 mm needle hydrophone with 4 dB and bandwidth of 0.2 - 15 MHz (Precision Acoustics LTD, Dorchester, UK). The PA signal acquired by the hydrophone is amplified by an ultrasound receiver amplifier and then digitized using an oscilloscope. The laser fluence was about 5 mJ/cm², and the PA signal was measured at a 100 different points in each well.

![Figure 1](image)

**Figure 1** block diagram of (a) combined US-PA imaging system and the gelatin tissue mimicking phantom with gold nanoparticle labeled cell inclusions, and (b) the set up used for measuring PA signal of gold nanoparticles labeled cells in 6 well plate

3. Results and Discussion

Three different sizes of AuNSs were synthesized and the UV-Vis spectra were obtained and the sizes were determined both by TEM and DLS. The UV-Vis spectra for gold nanospheres of different sizes are shown in Figure 2a. As the particles size increases, the absorption peak due to SPR is shifted to longer wavelength, the maximum wavelength $\lambda_m$ for the 12 nm, 66 nm and 90 nm nanoparticles are observed at 522, 538 and 556nm respectively. Furthermore, the peak width at the base is increased with increasing size which can be understood in terms of the fact that higher oscillation modes (quadrupole, octopole, absorption, and scattering) also affect the extinction cross section as the size increase[27,28]. The UV-Vis absorption spectra for citrate coated 12 nm Au spheres before and after PEGylation are shown in Figure 2b. It is observed that the SPR peak is red shifted about 3 nm after PEGylation, as indicated in the inset of Figure 2b which clearly indicates the PEG surface modification.
Figure 2 (a) UV-Vis Absorption spectra of Au Nanoparticles of the three different sizes, (b) UV-Vis Absorption spectra of the 12 nm Au Nanoparticles before and after PEGylation, a clear red shift of about 3 nm after PEGylation is shown in the inset.

The TEM image for two particle sizes (12nm, 66nm) are shown in Figures 3a and 3b respectively. The size distribution histogram for the smaller size is shown in Figure 3c having an average size of 11.8± 1.6 nm and for the larger size in Figure 3d having an average size of 65.92± 6.11 nm.

Figure 3 TEM images for two gold nanoparticles with different sizes (a) 11.8±0.81 nm, and (b) 65.92±5.32 nm, the size distribution for the ~ 12nm (c) and ~66 nm (d) respectively.

The hydrodynamic size for the AuNS was averaged over three measurements for each sample giving values for the particle sizes of 15.87 nm, 70.35 nm, and 98.06 nm with a poly dispersity index of about 0.08 as indicated in Figure 4 by a, b, and c respectively. Those values of hydrodynamic sizes are slightly different than the values obtained using TEM due to the presence of surfactant and the interaction with solvent molecules.
The Zeta potential measurements were carried out in order to confirm the surface modification of AuNS for citrate stabilized particles after and before PEGylation. The citrate coated AuNPs have a negative value of -34.3 mV as shown in Figure 5a. This is due to the attached citrate. After PEGylation, the Zeta potential is reduced to -9.35 mV as shown in b of Figure 5. AuNS of large size, obtained by addition of NH$_2$OH.HCl as a reducing agent, have a positive surface charge of about +19.8 mV their plot is represented in Figure 5c.

The US and PA images for the tissue mimicking phantom with cell inclusions loaded with citrate stabilized AuNPs are shown in Figure 6a on the left and right sides, respectively. Figure 6b shows the images for cells with PEGylated AuNPs. There is no difference between the US images for cells incubated with unPEGylated or PEGylated AuNS as the US image provides only a structural information about the tissue. While the PA image shows a very bright image for cells loaded with citrate stabilized AuNS, on the other hand there is almost no image in case of PEGylated AuNS. Such large increase in the PA signal may be due to the higher aggregation of citrate capped nanospheres on the cells than that of the PEGylated particles. This was also confirmed by the bright field microscope image taken for the cells loaded with the citrate shown in Figure 6c and PEG capped particles shown in Figure 6d. The two images were taken after incubation of the particles for the same period of time in media and at the same OD. Figure 6c shows a highly accumulated citrate capped nanoparticles on the
cells, whereas Figure 6d shows PEGylated particles of the same size at a much lower accumulation. As mentioned in the experimental part, AuNPs were incubated in culture medium that contains 10% Fetal Bovine Serum which results in binding of serum protein to the surface of nanoparticles. The binding of serum proteins results in surface modification and increasing intracellular uptake by receptor-mediated endocytosis[29, 30]. Cui et al [31] studied the interaction of different AuNPs with serum proteins. Their results show that citrate capped NPs are capable of adsorbing bovine serum albumin to form a 6-8 nm thickness protein corona. After modification with high densities of hydrophilic PEG, AuNPs have a high capacity to resist the adsorption of proteins. Therefore, these AuNPs circumvent cellular uptake. It should be mentioned that while the combined US-PA transducer scans the whole sample at once, the PA signal allows for taking measurements at different spots in the sample giving a large number of data to be averaged. The PA signal is measured for the cells while they are fixed in the 6 well cell culture plate, the signal is measured at 100 different points for each well to increase the precision. The PA signal for MDA-MB-231 Breast cancer cells loaded with 12 nm citrate stabilized AuNS, was first obtained at OD 0.5, 1, 3, and 4 and plotted as shown in Figure 6e. The PA signal for cells loaded with the same size but PEGylated spheres at the same OD was also plotted for comparison. It is observed that the citrate capped (unPEGylated) AuNs gives higher PA signal that is about 10 times more than the PEGylated particles for all ODs. This result explains the reduced cell uptake of the nanoparticles by MDA MB 231 breast cancer cells. Therefore it was decided to use unPEGylated particles in the rest of our study.

Figure 6 US (left) and PA (right) images for cells loaded with (a) 12 nm citrate stabilized and (b) PEGylated AuNPs, the US image in the left side, and the PA image in the right side, bright field microscope images for gold nanoparticles (c) capped with citrate, and (d) PEGylated AuNPs. (e) Variation of the PA signal with OD for cells
loaded with AuNPs capped with citrate, and PEGylated gold nanoparticle as indicated there is no observed PA
signal for PEGylated AuNS at any OD.

Furthermore, dark field microscope images were taken for the cells loaded with two different concentrations of
NPs but of the same size. Cells incubated with higher optical densities (OD 3) (Figure 7a) shows a higher light
scattering than cells incubated with particles of smaller optical densities (OD 1) (Figure 7b). The variations of
the PA signal with OD for three different sizes of AuNPs designated as small ~12 nm, medium ~66 nm, and
large ~90 nm are shown in Figure 8. For each particle size, there is an increase in the PA signal with the increase
in the OD. Furthermore, for the same amount of gold on the cells (same OD), it is observed that the PA signal
increase as the size of the AuNPs is decreased giving the highest PA signal for the smallest size 12 nm. This is
understood in terms of the increase of surface area for the smaller particle size. At the same OD 1, the surface
area for particle sizes of 10nm, 65 nm, and 90 nm are given by 17.9 cm², 2.9 cm², and 1.98 cm² respectively[32].
This surface area variation with the particle size agrees with the variation in the PA signal obtained within
experimental error. The aggregation of AuNPs causes the conduction electrons near each particle surface to
become delocalized and be shared amongst neighboring particles. This leads to the SPR to shift to lower energy
causing the absorption and scattering peaks to red shift[33]. Also, the aggregation may help in formation of more
hotspots for the smaller nanoparticles. With decreasing the particle size, the incident photons may undergo more
diffuse scattering within the sample which results in an increase in absorption and consequently the PA signal.

Figure 7 Dark field microscope image for cells after incubation with AuNPs with OD3 (a) and OD1 (b).
Figure 8 PA signal for cells loaded with gold nanoparticles with different ODs for three different particle sizes as indicated.

4. Conclusions

Using AuNPs as a contrast agent for PA imaging of MDA-MB 231 breast cancer cells, it is found that the PEGylation of nanoparticles reduce cell uptake than the citrate capped particles. This is because the PEGylated AuNPs have a higher capacity to resist the adsorption of proteins in the cell culture media which reduce the cellular uptake. Therefore, In order to employ PEGylated nanoparticles effectively, the nanoparticles have to be conjugated to a peptide to penetrate the cell to give a larger PA signal. The PA signal from AuNPs used as contrast agent, increases linearly with increasing the OD of NPs incubated with the cells. The PA signal obtained for cells loaded with AuNPs is significantly larger for smaller particles than larger size particles for the same OD as a result of the increase in their surface area. So, it is recommended to apply small AuNPs (~12 nm) as a PA contrast agent to obtain a more bright image with less amount of gold to avoid cell toxicity. The possible impact of the surfactant surface charge on the cell uptake of NPs and hence its effect on the PA signal is the subject of future study.

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