Gold nanoshell bioconjugates for molecular imaging in living cells

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Received October 4, 2004

Advances in scattering-based optical imaging technologies offer a new approach to noninvasive point-of-care detection, diagnosis, and monitoring of cancer. Emerging photonics technologies provide a cost-effective means to image tissue *in vivo* with high resolution in real time. Advancing the clinical potential of these imaging strategies requires the development of optical contrast agents targeted to specific molecular signatures of disease. We describe the use of a novel class of contrast agents based on nanoshell bioconjugates for molecular imaging in living cells. Nanoshells offer significant advantages over conventional imaging probes including continuous and broad wavelength tunability, far greater scattering and absorption coefficients, increased chemical stability, and improved biocompatibility. We show that nanoshell bioconjugates can be used to effectively target and image human epidermal growth factor receptor 2 (HER2), a clinically relevant biomarker, in live human breast carcinoma cells. © 2005 Optical Society of America

OCIS codes: 170.0170, 170.3880, 290.5850.

Optical imaging tools such as reflectance confocal microscopy (RCM) and optical coherence tomography (OCT) offer the potential for noninvasive, high-resolution *in vivo* imaging at competitive costs relative to current imaging modalities. Scattering-based optical technologies rely on inherent changes in indices of refraction for image contrast.[1] Strategies that depend on only the intrinsic optical contrast within tissue have proved clinically valuable in many screening applications including early cancer detection; however, such techniques are not sensitive enough to resolve an image based solely on the presence of biomarkers of disease. In cases of cancer, when early detection is critical to reducing morbidity and mortality, the use of molecular-specific contrast agents provides the capacity to optically sense and image abnormalities long before pathologic changes occur at the anatomic level. In addition, imaging based on molecular-specific targets allows real-time *in vivo* monitoring of the treatment course and can provide fundamental insights into cancer biology.[2] A recent demonstration of scattering-based optical molecular imaging used gold colloid conjugates to antibodies to the epidermal growth factor receptor as a contrast agent in imaging cervical cancer cells and biopsy samples.[3] Although gold colloid conjugates are highly valuable as contrast agents for detecting superficial epithelial cancers with visible light, there is particular need for contrast agents in the near-infrared (NIR) region of the spectrum. This is the spectral region in which tissue is most optically transparent,[4] allowing imaging of deeper tissue structures. The NIR region is also the region already exploited by RCM and OCT; thus contrast agents would provide greatly needed enhancement wherever these imaging modalities are utilized.

Over the years, the expanding availability of a variety of nanostructures with highly controllable optical properties has provided a series of new contrast agents for optical imaging. The use of a variety of nanomaterials such as quantum dots, gold nanoparticles, and their bioconjugates in biological imaging has been described in recent literature.[5,6] Typically, nanostructures have many properties far superior to molecular species such as Indocyanine Green. Advantages include higher quantum efficiencies, greater scattering and absorbance cross sections, optical activities over more biocompatible wavelengths, and significantly increased chemical or photochemical stability. Compared with Indocyanine Green, nanoshells provide a millionfold enhancement in optical extinction.[7] Nanostructures can also be targeted to specific molecular signatures of interest. Therefore the systematic control of nanostructure properties that can be obtained by particular size variations is in direct contrast with molecular probes, whose properties vary nonsystematically between molecular species.

Nanoshells are a novel class of nanoparticles composed of a dielectric silica core surrounded by a thin metallic shell, which is typically made of gold. Nanoshells have a strong optical resonance whose wavelength can be tuned across much of the visible and infrared region of the spectrum by varying the relative size of the core and shell layer.[8] Varying the absolute nanoparticle size allows the relative contributions of scattering and absorption at a given wavelength of interest to be controlled.[9] This extremely agile tunability of the optical resonance is completely unique to nanoshells. Gold nanoshells are capable of scattering light in the NIR and provide appealing optical properties for use in conjunction with reflectance-based optical imaging methods. Additionally, the gold surface is biologically inert and allows proteins to be readily conjugated, facilitating *in vivo* use.[10]

Nanoshells have demonstrated promise in a variety of biomedical applications ranging from substrates for whole-blood immunoassays[11] to photothermal cancer therapy. By use of magnetic resonance thermal guidance, *in vitro* cancer cells were successfully ablated with gold nanoshells tuned to absorb

0146-9592/05/091012-3/$15.00

NIR light.[12] Similar use of nanoshells for photothermal ablation of tumors in mice further showed complete regression of tumors with the mice remaining healthy compared with controls.[13] In contrast with therapeutic NIR-absorbing nanoshells, we fabricate highly scattering NIR nanoshells for optical imaging. We then demonstrate the feasibility of using these targeted nanoshell bioconjugates as contrast agents to image human epidermal growth factor receptor 2 (HER2) expression in living human breast carcinoma cells.

HER2-positive SKBr3 human breast cancer cells were cultured in McCoy's 5A modified medium supplemented with 10% fetal bovine serum and antibiotics. HER2-negative MCF7 human breast cancer cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 0.01 mg/ml of bovine insulin, and antibiotics. Cells were maintained at 37°C and 5% CO_2.

The synthetic protocol developed for the fabrication of gold nanoshells is based on the principles of molecular self-assembly and colloid chemistry in aqueous solution. On the basis of the Stöber method,[14] we fabricated silica nanoparticle cores by reducing tetraethylorthosilicate in ammonium hydroxide and ethanol. Particle surfaces were terminated with amine groups by reaction with aminopropyltriethoxysilane. Small gold colloid was grown with the method of Duff and Baiker.[15] Gold–silica nanoshells were then grown by reacting gold salt ($HAuCl_4$) with the silica–colloid particles in the presence of formaldehyde. Nanoshell formation was assessed with an UV–Vis spectrophotometer and scanning electron microscopy. Nanoshell dimensions were mathematically assessed with Mie scattering theory with good agreement with scattering electron microscopy and UV–Vis spectra.

Either anti-HER2 (specific) or anti-immunoglobulin G (anti-IgG) (nonspecific) antibodies were attached to a polyethyleneglycol (PEG) linker [orthopyridyldisulfide-polyethyleneglycol-*N*-hydroxysuccinimide (OPSS-PEG-NHS), molecular weight of 2000] through a hydroxysuccinimide group (NHS). The antibody–PEG linker complex was then attached to the nanoshell surface through a sulfur-containing group located at the distal end of the PEG linker. By use of $NaHCO_3$ (100 mM, pH of 8.5), OPSS-PEG-NHS was resuspended to a volume equal to that of the antibody. The reaction was allowed to proceed on ice overnight. Excess unbound polymer was removed by membrane dialysis (molecular weight cutoff of 10,000). PEG-ylated antibody (0.67 mg/ml) was added to nanoshells (2×10^9 nanoshells/ml) to facilitate targeting. After antibody conjugation, nanoshell surfaces were coated with PEG-thiol (PEG-SH, molecular weight of 5000, 25 mM) to block nonspecific adsorption sites.

HER2-expressing SKBr3 cells were exposed to bioconjugated nanoshells (8 μg/mL) and observed under dark-field microscopy, a form of microscopy sensitive only to scattered light. Images were taken with a Zeiss Axioskop 2 plus microscope equipped with a black-and-white CCD camera under the same magnification and lighting conditions. Optical contrast was quantified with the Scion image analysis program. Average intensity values were obtained in each dark-field image. Normality of intensity data was established through a Shapiro Wilk test before using a paired Student's t-test (two tailed) to test for significance.

Figure 1 shows the optical properties for nanoshells with a 120-nm silica core radius and 35-nm-thick shell that were used in this study. Nanoshells with these dimensions generate a scattering spectrum beginning at 700 nm and extending far into the NIR region; thus nanoshells with these spectral characteristics are capable of facilitating imaging in both the visible and the NIR regions, allowing the nanoshell conjugates to be used as contrast agents for RCM and OCT.

As shown in Fig. 2, significantly increased optical contrast under dark-field conditions caused by HER2 expression was observed in HER2-positive cells targeted with anti-HER2-labeled nanoshells (right column) compared with cells targeted by either anti-IgG-labeled nanoshells (middle column) or cells not exposed to nanoshell conjugates (left column). Images in Figs. 2(a)–2(c) are cross-sectional slices of cells taken at the mid-focal plane at 40× magnification. A series of dark-field images [Figs. 2(d)–2(f)] taken at a lower magnification (10×) is included to demonstrate nanoshell targeting and coating of cell surfaces [Fig. 2(f)].

Histogram analysis of dark-field images shows that nanoshell targeting of the HER2 receptor resulted in significantly ($p < 0.05$) greater average contrast values in the anti-HER2 group (142±16) compared with controls (anti-IgG 48±12, no nanoshells 26±4) (Fig. 3). Significantly less contrast was measured in HER2-negative cells exposed to anti-HER2-labeled nanoshells (34±5) compared with HER2-positive cells (142±16), providing additional evidence that the increased contrast seen under dark-field conditions may be specifically attributable to nanoshell targeting of the HER2 receptor. No significant differences were found between control groups.

Knowledge of potential molecular targets for diagnosis and therapy of disease continues to expand at a

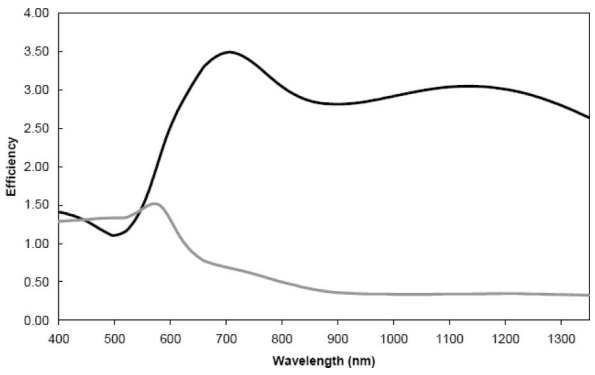

Fig. 1. Mie scattering theory predictions of the scattering (black) and absorption (gray) efficiencies for nanoshells with the 120-nm silica core radius and 35-nm-thick shell that were used in this study.

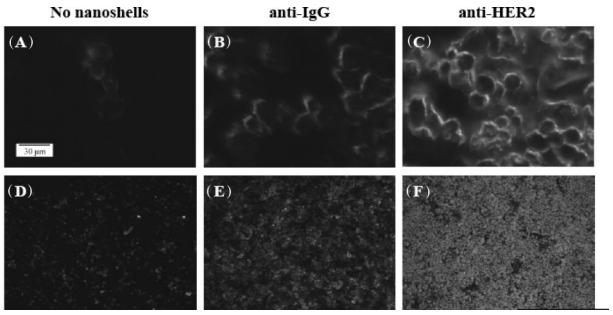

Fig. 2. (a)–(c) High-magnification dark-field images of HER2-positive SKBr3 breast cancer cells exposed to no nanoshells (left-hand column), anti-IgG-labeled nanoshells (middle column), or anti-HER2-labeled nanoshells (right-hand column). Cross-sectional images were taken at 40× magnification at the mid-focal plane. (d)–(f) Dark-field images of HER2-positive cells taken at lower magnification (10×) demonstrating nanoshell targeting and coating of the cell surface.

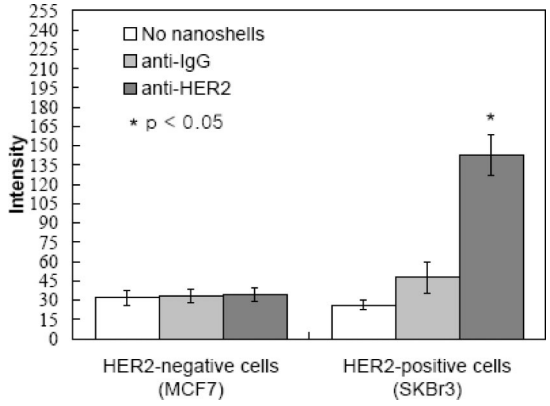

Fig. 3. Quantitative analysis of optical contrast owing to HER2-expression. Contrast was quantified by obtaining average histogram intensity values of dark-field images. Contrast data quantified with a HER2-negative MCF7 cell line are shown for comparison purposes. Intensity values range from 0 (black) to 255 (white), with higher values corresponding to greater contrast. Differences in mean scattered intensity between the anti-HER2 group and all other cell groups are statistically significant ($p < 0.05$).

rapid rate. However, translating knowledge of potential targets into new diagnostic and therapeutic techniques requires the development of methods to image molecular targets or the effects of therapeutic interventions on these targets *in vivo*, in real time, and in a cost-effective manner. Nanoshell-based molecular contrast agents offer unique advantages, including NIR tunability, size flexibility, and systematic control of optical properties. In this study we demonstrated that nanoshell bioconjugates can provide molecular optical contrast enhancement both qualitatively and quantitatively. Our findings collectively show that gold nanoshells can be used to target specific cancer markers and allow *in vitro* cell-level molecular imaging with a scattering-based optical approach. A dark-field microscope was used in this study to demonstrate the feasibility of nanoshell bioconjugates for molecular imaging in living cells. Although dark-field microscopy is appropriate for *in vitro* imaging applications, use of nanoshell conjugates *in vivo* will require more sophisticated imaging techniques. Our current results encourage future work assessing nanoshell-based contrast agents *in vivo* with RCM and OCT. The combination of targeted nanoshells and the field of biophotonics have the potential to play a vital role in the future of cancer screening and diagnosis, in designing and monitoring therapeutic interventions, and in fundamental studies of carcinogenesis.

Funding for this project was provided by the National Science Foundation (BES 022-1544), the National Science Foundation Center for Biological and Environmental Nanotechnology (EEC-0118007), and the Department of Defense Congressionally Directed Medical Research Program (DAMD17-03-1-0384). C. Loo's e-mail address is cloo@rice.edu.

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