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Nanoshells for *In Vivo* Imaging Using Two-photon Excitation Microscopy

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Abstract

Gold nanoshells have been intensively investigated and applied to various biomedical fields because of their flexible optical tunability and biological compatibility. They hold great potential to serve as luminescent contrast agents excitable with near infrared (NIR) lasers. In this paper, we describe the development of nanoshells with a peak of plasmon resonance at 800 nm and their subsequent use for *in vivo* blood vessel imaging using two-photon excitation microscopy at an excitation wavelength of 750 nm. We were able to image single nanoshell particles in blood vessels and generate optical contrast for blood vessel structure using luminescent signals. These results confirm the feasibility of engineering nanoshells with controlled optical properties for single-particle-based *in vivo* imaging.

Keywords

Gold nanoshells; Luminescent; Two-photon Excitation Microscopy

1. Introduction

Metal nanoshells represent a class of nanoparticles consisting of a spherical dielectric core with a thin metallic shell [31]. Among various types of metal nanoshells, gold-coated nanoshells are widely used in biomedical applications because they are biocompatible [7, 26]. By varying both the thickness of the gold layer and the particle size, optical resonance of the nanoshells can be fine-tuned to wavelengths ranging from the visible into the NIR spectrum [21, 15, 23], the region of highest physiological transmissivity [32, 5]. Because of these unique properties, nanoshells have been used as NIR absorbers to efficiently transfer photonic energy into heat, resulting in their broad application in drug delivery [36] as well as cancer therapy [6, 18, 17].

Because of their scattering and absorption properties, nanoshells are also frequently used in other imaging applications, such as scattering-based optical coherence tomography [14, 24] and luminescence-based optical microscopy [3, 34]. Compared to other scattering-based imaging techniques, which rely heavily on the inherent variation in refractive indices of the tissue in order to provide contrast [20, 13], a luminescence-based microscopy system can better reveal the object of interest in an otherwise dark background [25]. For example, two-photon microscopy [9] provides exceptional optical sectioning capability and target-to-background contrast at sub-micron resolution [16, 25, 11]. As such, it presents an ideal

platform for small animal high-resolution imaging and molecular targeting [10, 41, 16]. However, because of the strong scattering of the excitation light, the maximum imaging depth ever shown *in vivo* has only been about 600–1000 μm in the neocortex of both mice and rats [40, 30, 37, 38]. The use of nanoshells with this technique results in much higher luminescence intensity compared to contrast agents of similar size [33, 34]. This brighter optical contrast can, in turn, potentially extend current imaging depth of two-photon microscopy.

This study employs a two-photon microscopy platform to image gold nanoshells at a submicrometer scale, exploiting the luminescent properties of nanoshells as well as their resulting utility for *in vivo* imaging in live tissue or organisms. Luminescence from single nanoparticles is resolved to generate useful optical contrast. Specifically, single nanoshells were successfully identified *in vivo* while flowing through a blood vessel, demonstrating that optical contrast can be attributed to a single nanoshell in blood vessel imaging. These results illustrate considerable potential for nanoshells that have been engineered to exhibit specific optical properties suited for *in vivo* imaging. Such nanoshells will improve the imaging depth of two-photon excitation microscopy, thus offering a new approach to high-resolution optical imaging in combination with cancer therapy.

2. Methods and Materials

2.1 Nanoshell synthesis and characterization

The fabrication protocol used for nanoshells includes molecular self-assembly and colloid chemistry in aqueous solution [45]. Silica cores were made by the Stöber method, which involves the reduction of tetraethylorthosilicate (TEOS, Aldrich) in ammonia hydroxide ethanol. Next, the Stöber nanoparticles were functionalized with aminopropyltriethoxysilane (APTES, Aldrich), resulting in silica cores with attached amine groups. When these functionalized groups were placed in a gold colloid solution, small gold colloids adsorbed to the amine groups. These small gold colloids (1–3 nm) were fabricated according to the method of Duff and Baiker [12]. Gold colloids provide the nucleation sites for further reduction of gold. As gold reduces onto the colloid, the colloids grow until the complex coalesces into a complete shell. The amount of gold added during this final reduction stage determines the shell thickness. Gold nanoshell sizes were determined by scanning electron microscopy (SEM), and their absorbance was measured with a spectrophotometer (Cary 5000, Varian). Before the experiments, nanoshell surfaces were modified with thiolated polyethylene glycol (PEG) to provide steric stabilization as well as to eliminate nonspecific protein adsorption [27].

2.2 Luminescent spectrum of nanoshells

A drop of PEGylated nanoshell dispersion at a concentration of 3.5×10^9 particles/ml was dried on a glass slide and was then imaged with a Zeiss Plan-Apochromat 20X, NA=0.75 objective lens using a two-photon excitation microscope (Zeiss, 510 META) coupled to a Coherent Chameleon tunable titanium:sapphire laser (Coherent Laser Group, Santa Clara, California). The laser was tuned to 750 nm and 800 nm for excitation. Laser power at the specimen was about 2 mW. The emission signal was collected from 380 – 640 nm using a built-in spectrometer (META) with a spectral resolution of 20 nm. The META detector can be used to collect images at different wavelengths simultaneously. The signals used in the measurement of the spectrum came from single pixels in the image at 1X zoom where the pixel size was 850 nm. Since this size was much larger than the size of a single nanoshell, it was possible that emission signals came from particle aggregates. In fact, nanoshells often show the tendency to cluster upon drying. To further characterize the imaged particles as either single particles or particle aggregates, a higher digital magnification using the same

lens was employed to image the same particles, but using a smaller pixel size. Nanoshell clusters owing to their larger size compared to a single nanoshell need more pixels to cover the spatial extent of the cluster. Thus as the digital zoom is increased signal from a single nanoshell will be confined to a single pixel while that from a cluster requires more than one pixel depending on the size of the cluster. However, it is not possible to resolve the individual nanoshell in the cluster even at large digital zoom since it does not improve the lateral optical resolution. Most of these signals were confined to a single pixel size of 250 nm at 3.5X magnification. Under these conditions, the pixel size was comparable to the size of a single nanoshell; particle aggregates should occupy more than one pixel. Based on this observation, we conclude that the emission spectrum comes from single nanoshells, not nanoshell clusters. Regions in the images without nanoshells were sampled as background. The background intensities were subtracted from the original measurements to extract the emission data of nanoshells.

2.3 Ex vivo imaging of nanoshells using two-photon excitation microscopy

PEGylated nanoshells were suspended in a trehalose solution (2.33×10^{10} particles/ml) for tail vein injection. 150 μ l of the nanoshell solution was injected, along with 50 μ l of a fixable dextran conjugated with Oregon Green 514 (25 mg/ml, 70,000 MW, 2.15×10^{20} molecules/ml, Invitrogen), into the tail vein of two CD1 mice. Quadriceps muscles of mice were sectioned 30 minutes after injection for *ex vivo* imaging. Two control mice received an injection containing only nanoshells (at the same concentration). Tissue sections were fixed with 4% PFA and were stained with Hoechst (Hoechst 33342, Invitrogen). Twenty slides from each mice were imaged with a custom-built Zeiss two-photon excitation microscope using a Zeiss C-Apochromat 32X, NA=0.85W objective lens. Nanoshells, dextran, and Hoechst were excited using 750 nm, 850 nm and 720 nm lasers, respectively. Laser power at the specimen was about 10 mW. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine.

2.4 In vivo imaging of nanoshells using two-photon excitation microscopy

150 μ l of the same nanoshell solution described in Sec. 2.3 was injected, along with 50 μ l of fixable dextran Oregon Green 514 (25 mg/ml, 70,000 MW, 2.15×10^{20} molecules/ml, Invitrogen), into the tail vein of two CD1 mice. Two mice injected only with 50 μ l of fixable dextran Oregon Green 514 (25 mg/ml, 70,000 MW, 2.15×10^{20} molecules/ml, Invitrogen) were used as the control. Through local surgery, veins in the quadriceps muscles of the anesthetized mice were exposed and then imaged with a custom-built Zeiss Axioskop 2FS upright two-photon excitation microscope, mounted with a modified LSM DuoScan system, using a Zeiss Plan-Apochromat 20X, NA=1.0 objective. A titanium:sapphire laser (Coherent Laser Group, Santa Clara, California) was tuned to 750 nm to excite the nanoshells and fluorescent dextran with a power of about 20 mW at the specimen. Five to ten time series (10s, 1s interval) were taken from each mouse.

2.5 Ex vivo imaging of nanoshells using scanning electron microscopy (SEM)

After the *in vivo* imaging, quadriceps muscles of the mice were embedded in paraffin and sectioned for SEM imaging *ex vivo*. Tissue sections were first rehydrated in water and then dehydrated with a graded series of increasing concentrations of ethanol for 5 minutes each. The samples were then transferred to a graded series of increasing concentrations of hexamethyldisilazane (HMDS) for 5 minutes each and air-dried overnight. In the next step, the samples were first coated with flash carbon under vacuum using a Balzers MED 010 evaporator (Technotrade International, Manchester, NH) and then transferred to a desiccator for the dehydration process. Finally, the samples were imaged using a JSM-5910 scanning electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 15 kV.

One slide from each mice was imaged and five pictures were taken from each slide, resulting in ten SEM images for both the nanoshell and control group.

3. Results

3.1 Nanoshell fabrication and characterization

The nanoshells used in this experiment have an average core size of 132 nm in diameter and an average shell thickness of 23 nm. The overall diameter of these nanoshells is 178 nm. The SEM images of the nanoshells are shown in figure 1(a). The extinction spectrum of the nanoshells used in this study was theoretically calculated using Mie theory based program known as G-shell [39, 35, 31] (Courtesy of Dr. Naomi Halas, Rice University). Following parameters were used for calculation of the extinction spectrum: the dielectric constant of core (SiO_2)-2.04, of the medium (H_2O)-1.77, and of the Au shell [22] as well as the radius of the silica core -66 nm and the thickness of the gold shell-23 nm. Figure 1(b) shows that the measured extinction spectrum matches the simulated results, giving an extinction peak at 800 nm.

3.2 Luminescence spectrum of nanoshells

Figure 2 shows the emission spectrum measured from 380 to 640 nm after background subtraction. The emission spectrum intensity continually increased as the emission wavelength increased up to 640 nm. The data points measured at 660 nm were not used since 660 nm was on the edge of the KP 660 emission filter. The global maximum in this range occurred at 640 nm, which is in the red region of the visible spectrum. Luminescence signals from the nanoshells show a similar spectral pattern whether excitation is done at 750 nm or 800 nm.

3.3 Ex vivo imaging of nanoshells

Nanoshells were first imaged *ex vivo* in tissue sections to provide information, such as density and distribution, necessary for their *in vivo* detection. Figure 3(c) shows scattered nanoshells (white arrows) inside a vessel in a tissue section after excitation at 750 nm. Using 800 nm excitation and a 500–550 nm filter, a vessel lumen was labeled with fixable dextran (figure 3(b)). The control case shown in figure 3(f) indicates that the scattered pattern exhibited by the nanoshells is not due to the presence of dextran. Based on these results, it was expected that nanoshells would be visible as bright, scattered particles for *in vivo* detection and that the luminescence from these particles would originate from nanoshells, not aggregated dextran. It is worth mention that the size of a single pixel when using a 32x objective is around 530 nm by 530 nm. The mean diameter of the nanoshells used here is 178 nm. Therefore, the signal from the entire nanoshell is restricted to a single pixel unless they aggregate significantly. The noise signal in the image is also confined to a single pixel. This effect has caused the lower contrast of nanoshells in the original images than in the zoomed insets.

3.4 In vivo imaging of nanoshells using two-photon excitation microscopy

Based on *ex vivo* observations, nanoshells were imaged *in vivo*. Flowing particles were observed within dextran-filled vessels (figure 4(a–c)), and they showed a scattered distribution similar to the *ex vivo* results. The particles were absent in the control mouse, which received an injection of dextran alone and was imaged with the same configurations (figure 4(f–h)). Luminescence emission from nanoshells was collected from 520 nm to 640 nm with band-pass filters of about 50 nm in width. The emission intensity increased with the emission wavelength. The maximum emission intensity occurred when using a band-pass filter from 575 nm to 640 nm (BP 575–640), which was well within the predetermined

emission pattern of nanoshells. Importantly, the PEGylation test showed a less than 5% drop of O.D. over 30 minutes, which indicated that the tested PEGylated nanoshells did not aggregate in the PBS solution. The brightness of the nanoparticles distinguished them from the dextran signal. Figure 4(d) and (i) are the isolated images from (c) and (h), respectively. Pixel intensity from each channel (520–560 nm, 575–640 nm) ranged from 0–255. An intensity threshold of 250 was used for both channels to isolate nanoshells from the original vessel images. The same threshold was used for the control images. The isolated nanoshells occupied single pixels. To achieve better visualization of the nanoshells, each of the pixels occupied by the nanoshells were dilated to include their eight adjacent neighbors. Figure 4(d) shows isolated nanoshell signals in the vasculature, and the control image (figure 4(i)) shows little background using this method.

3.5 Single particle analysis

In figure 4(a–c), bright particles were imaged while flowing inside vessels. In order to identify these particles as single nanoshells or nanoshell aggregates, the particle concentration in circulation was analyzed. On average, the total blood volume in a mouse is 6–8% of its body weight, or 6–8 ml of blood per 100 g of body weight [19]. Thus, for a 20 g lab mouse, the total blood volume is 1.40 ± 0.20 ml. Given 2.33×10^{10} particles/ml as the injection concentration for nanoshells, the estimated blood concentration for single nanoshells would be $2.19 \pm 0.27 \times 10^9$ particles/ml, assuming a uniform distribution of the particles inside the vasculature and no clearance of particles from the blood after injection. In order to compare the calculated result with the experimental observation, 30 nanoshell pictures (similar to figure 4(c)) and five control pictures (similar to figure 4(h)) were used to calculate the particle density in the vessels. They were isolated in a manner similar to those in figure 4(d) and (i). The number of particles was calculated after isolation, and the areas of the vessels were measured with Zeiss LSM Image Browser software using dextran-labeled vessels. The average background level from dextran in the control case was 8.10×10^{-5} particles/ μm^3 , which was used as a baseline for the nanoshell density calculation. In two-photon excitation microscopy, the excitation volume is on a sub-femtoliter (fl) level (0.1–0.5 fl) [10], which corresponds to an imaging depth of about 1 μm . Our measurement determined that the average density of nanoshells was 1.71 ± 0.21 particles/ μm^3 , which equals $78.01\% \pm 13.72\%$ of the calculated value. Considering that the PEGylation test showed a drop in O.D. of less than 5% over 30 minutes in PBS, this measurement suggests that nanoshell aggregation was, in fact, not a recurring problem *in vivo* and that the flowing particles observed *in vivo* could be considered single nanoshells.

To test the single-particle hypothesis, tissue sections were imaged with SEM (Figure 5), and the particle sizes were measured using 5,000 and 10,000 magnifications. Nine SEM images (a total of 232 nanoshell particles) from the nanoshell injected mice was used for this calculation. Measurements from SEM images further showed that $(89.83 \pm 15.41)\%$ ($n = 9$ SEM images) of the nanoshell particles have a size ranging from 160 nm to 200 nm, which is within the size range of a single nanoshell. Therefore, we conclude that the particles imaged *in vivo* were mainly single particles. Two possible reasons explain why only 78.01% of the theoretical particle density could be observed in the experimental data. First, although *in vivo* imaging was conducted within a short time span (less than 30 minutes) after injection, it is possible that some particles were cleared out of the blood system and accumulated in various organs (e.g. spleen and liver) [42, 8]. Second, it is possible that not all of the particles were visible when imaged with the microscope; this is especially true for those nanoshells that were hundreds of micrometers beneath the tissue surface.

3.6 Contrast for vessels

To compensate for the low density of nanoshells inside the vessels and provide better contrast for vessel structure, a time series reconstruction algorithm was employed. Such a method has previously been utilized to reconstruct a bifurcation structure [1, 2]. In our case, reconstruction from a time series acquired at the same place generated a higher density of particles in the composite image. Figure 6(a) shows one image from a 10s long time series; figure 6(b) shows isolated nanoshells from this image; and figure 6(c) shows a reconstructed image from the time series. The nanoshells in each image were isolated using the same methodology described for figure 4(d). Ten pictures from the time series were used to reconstruct figure 6(c). Using this method, a higher density of visualized nanoshells is accessible to improve contrast for *in vivo* vessel imaging

4. Discussion

Gold-silica nanoshells were engineered with an extinction peak in the NIR region. Using a two-photon excitation microscope, the luminescent spectrum of the nanoshells was determined to have a broad range of emission, increasing as wavelength increased up to 640 nm. The spectrum pattern agrees with previous reports on the luminescence spectra of similar nanoparticles [29, 44, 4, 43]. This is beneficial for NIR imaging of tissues since longer-wavelength emission light undergoes less scattering and has greater penetration depth. In the *in vivo* experiment, bright flowing particles were observed together with fluorescent dextran (figure 4), while the control samples with only dextran did not exhibit any visible contrast from flowing particles. To further characterize the particles, a concentration analysis was performed. The density of the flowing particles observed in the *in vivo* experiment accounts for 78.01% of the maximum possible single particle density, assuming that all particles were uniformly distributed in the vasculature after injection and that no particles were cleared out of the blood system. Further SEM imaging showed that around 89.83% of the particles in the tissue section remained as single particles. Therefore, it can be concluded that the flowing particles were mainly single particles, not clusters or aggregates. To the best of our knowledge, this is the first report showing the feasibility of using nanoshells for single-particle-based *in vivo* imaging. The single-particle approach is attractive since the nanoshells used for *in vivo* imaging can be easily characterized *ex vivo*. Compared to particle aggregations, whose optical properties are complex and difficult to predict owing to irregularities of particle accumulation, single particles behave in a controlled way between experiments, which is elucidated by Mie theory [28] and can be analyzed consistently. Additionally, the single-particle approach possesses much broader imaging applications, such as the use of molecular targeted nanoparticles to detect the presence, relative abundance, distribution, and even transportation of the bio-molecules in their micro environment.

While nanoshells appeared to exhibit brighter emission than dextran, the low density of nanoshells observed inside the blood vessels could potentially have a negative impact on their utility for vessel imaging as single particles. Use of more concentrated particle solutions or increased injection volume can potentially alleviate this problem, but could also lead to disruption of vessels possibly due to heating of nanoshells. To address this problem, time series reconstruction was used. Time series were acquired at the same locations using a low concentration of nanoshells. The nanoshells from each image were isolated and combined to provide contrast for the vessel (figure 6). Using this method, it is even possible to monitor structural changes of the vasculature using a long-term imaging process [1, 2]. In addition, the combination of targeted nanoshells with time series images could be another effective approach for future research. More specifically, nanoshells can be targeted by surface coating of antibodies or polymers to target the tissue of interest, such as vessels or tissue cells. This technique selectively accumulates nanoshells around the region of interest

in order to achieve the needed level of contrast between the target and the background. At the same time, with an increased population of nanoshells specifically labeling the region of interest, the required laser power is consequently reduced, and the chance of photothermal damage during an imaging session is therefore minimized. For deeper tissue imaging, combining a longer imaging acquisition time with the reconstruction of the time series images is a good choice to enhance the contrast and the signal-to-noise ratio. This imaging approach can be further combined with therapy. After the imaging, photothermal therapy can be executed with the same type of nanoshells, eliminating the cost and time of administering additional types of therapeutic agents. Concurrently, this could be further exploited as a single-particle-based tracking system for drug delivery and for monitoring the accumulation of nanoshells in cancer tissue for photothermal therapy.

5. Conclusions

The feasibility of using nanoshells for single-particle-based *in vivo* imaging has been validated in this study. First, using a NIR high-resolution imaging platform, monodispersed nanoshells were imaged in live animals, resulting in a useful optical contrast. Next, and importantly, a majority of the particles were characterized as single nanoshells through SEM imaging, demonstrating a novel imaging strategy capable of resolving single nanoshells *in vivo*. Finally, vessel structures were successfully reconstructed from time series by utilizing a reconstruction algorithm to increase the density of the nanoshells in the final image. Therefore, our study suggests that gold-silica nanoshells with tunable optical properties hold considerable potential in molecular imaging and can be further developed into a new class of contrast agents for NIR luminescent imaging.

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Figure 1.

(a) SEM image of the nanoshells. Nanoshells are 178 nm in diameter with a core size of 132 nm and a shell thickness of 23 nm. (b) Measured extinction spectrum and simulated results using G-shell are shown with blue and red curves, respectively.

Figure 2.

Luminescent spectra of nanoshells used in this study. Data points from 750 nm and 800 nm excitation are shown in blue and red, respectively. Background signals are also shown, representing the baseline. Nanoshell emission data were calculated by subtracting these background signals from the original measurements.

Figure 3.

Ex vivo imaging of nanoshells in a tissue section. Images in (a)–(c) are from mice receiving an injection containing both dextran and nanoshells. Images in (d)–(f) are from control mice receiving an injection containing only nanoshells. (a) and (d) Cell nuclei labeled with Hoechst. (b) Vessel lumen labeled with fixable dextran. (e) Tissue autofluorescence. (c) and (f) show nanoshell signal in the vessel (white arrows). Regions of interest (white rectangles) were amplified and displayed in the upper right panels. Cell nuclei are visible because Hoechst emission extends into the 575–640 nm wavelength range when excited with a 750 nm laser.

Figure 4.

In vivo imaging of flowing nanoshells in the veins of a mouse quadriceps muscle using two-photon excitation microscopy. (a),(b) Emission signals in the wavelength range from 575–640 nm and 520–560 nm, respectively. (c) Composite image created by merging (a) and (b). (e) Amplified image from the region of interest in (c). In these images, flowing particles (white arrows) created brighter signals than dextran. The primary emission peak of dextran labeled with Oregon Green 514 is in the 520 – 560 nm region, but its emission does extend into 575 nm–640 nm region. (f), (g) Control mice which were injected with only labeled dextran and were imaged with the same imaging settings. (h) Composite image created by merging (f) and (g). (j) Amplified image from the region of interest in (h). Brighter nanoshells were isolated from the dextran by thresholding. (d), (i) Isolated images from (c) and (h), respectively. The same threshold value was used in both cases. Blood cells (blue arrows) appear as black spots in images of blood vessels injected with Dextran.

Figure 5.

SEM images of tissue sections after *in vivo* imaging. (A)–(C) and (D)–(F) show two blood vessels with increased magnifications from left to right, respectively. The bright particles are nanoshells. (D)–(F) illustrate a representative case where nanoshells and blood cells are confined within a vessel. In (E) and (F), nanoshells are clearly observed in the vicinity of blood cells as single particles.

Figure 6. Optical contrast for a vessel reconstructed using a time series from flowing nanoshells. (a) One picture in a time series used for reconstruction. (b) Nanoshells isolated from (a). (c) Reconstructed image using ten images from this time series.