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Three-Dimensional High Resolution Imaging of Gold Nanorods Uptake in Sentinel Lymph Nodes

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Abstract

In this paper, we demonstrate an application of a non-invasive imaging modality, photothermal optical coherence tomography (PT-OCT), for imaging gold nanorods (GNRs) uptake in sentinel lymph node (SLN) of mice *in situ*. This application enables us to obtain higher quality images of SLN structures due to the photothermal contrast properties of the GNRs. It is also demonstrated that GNRs accumulate differently within several SLN structures, and this uptake is time dependent. Finally, we determine the average concentration of GNRs within the whole SLN which is used to demonstrate uptake kinetics of the nanoparticles.

Keywords

Gold nanorods; sentinel lymph node; photothermal; optical coherence tomography

Introduction

Sentinel lymph nodes (SLN) are the first draining lymph nodes that are reached by metastatic cancer cells from a primary tumor. The SLN of cancer suspects are biopsied and sent to a pathologist to determine the presence of cancer cells.¹ A cancer-free SLN is an indicator that the cancer has not metastasized beyond that lymph node; therefore, no more lymph nodes are dissected. The standard of care requires pathologists to observe histological samples of the SLN, which requires destroying the samples. Current imaging techniques can obtain pictures of the SLN non-invasively. These imaging techniques have been clinically used to map the location of SLN *in vivo*; such as, magnetic resonance imaging and computed tomography.^{2, 3} The success rate of these imaging techniques has been enhanced by using various contrast agents such as radioactive technetium-99 (^{99m}Tc) colloids, iron oxide nanoparticles and quantum dots.^{2, 4, 5} Recently, photoacoustic (PA) imaging has also been demonstrated to map the location of SLN's *in vivo* using nanoparticles as a contrast agent.^{6, 7} Although these techniques are useful in identifying the location of the SLN, they have poor resolution for imaging structural details with a quality comparable to histological samples. Most recently, optical-resolution photoacoustic microscopy has been developed that delivers a spatial resolution at sub-micron scale.⁸ However, its capability to delineate the structural details of SLN has yet to be confirmed.

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Optical coherence tomography (OCT) is a non-invasive tool used to obtain three dimensional (3D) cellular and subcellular tissue morphology with micrometer-scale resolution using the coherence-gated detection of scattered light.⁹ OCT has recently been used to provide images of lymph node morphology *ex vivo*.¹⁰ The reported OCT images depict morphological structures that corresponded well with histological features of the lymph nodes (LN), suggesting the potential of using OCT to visualize lymph node microstructures on a scale of micrometastases, and to detect metastatic nodal diseases intraoperatively. However, these morphological studies detected no significant differences between the OCT images of LNs from tumor-bearing animals and those from control animals. Photothermal OCT (PT-OCT), a variation of the OCT technology, is sensitive to optical phase changes that are induced on the tissue by changes in temperature.¹¹ Contrast agents that increase their temperature when absorbing light (photothermal effect) are suitable for PT-OCT. Gold-based contrast agents are attractive for PT-OCT because they are biocompatible, their absorption and scattering properties are customizable, and the gold surface can be conjugated with antibodies and peptides that bind selectively with proteins associated with specific diseases, such as cancer.¹² Among them, gold nanorods (GNRs) can be designed to have high absorption with a narrow spectral bandwidth in the near-infrared (NIR) region, an optical window where biological tissues have low absorption, by controlling its aspect ratio.¹³

PT-OCT has been used to obtain high resolution images of *ex-vivo* human breast tissue.¹⁴ A variation of this method has also been used for photothermal cancer therapy in a mouse model.¹⁵ In this study, we demonstrate the use of GNRs as a contrast agent for three-dimensional imaging of mouse SLN *in situ* using a PT-OCT system. This system is sensitive to detecting the uptake of GNR's at different SLN structures. We have also obtained SLN images at different time points, demonstrating the potential of using this technique to study SLN functionality by observing the dynamic migration of GNRs through different tissue structures.

Experimental Methods

Gold Nanorods

GNRs were prepared using a seed-mediated growth technique which provides control over its size and aspect ratio.¹⁶ The process for preparing the GNRs is summarized in Figure 1a. Cetyltrimethylammonium Bromide (CTAB) solution (2.5 mL, 0.20 M) was mixed with 1.0 mL of 0.001 M HAuCl₄. This solution was then mixed with 0.40 mL of ice-cold 0.010 M NaBH₄, which resulted in the formation of a brownish yellow seed solution. The seed solution was stirred vigorously for 2 min, and then it was kept at a temperature of 45 °C for 5 min. A growth solution was prepared separately by combining 5 mL of 0.20 M CTAB with 0.25 mL of 0.0040 M AgNO₃ at room temperature. Subsequently, 0.5 mL of 0.01 M HAuCl₄ was added to the mixture, and after gentle mixing of the solution, 70 µL of 0.0788 M ascorbic acid was added. The color of the growth solution changed from dark yellow to colorless. The growth solution was mixed with 12 µL of the seed solution and kept for 20 min at room temperature. The solution was centrifuged, decanted and re-dispersed in water several times to remove excess CTAB, which is toxic. Finally, the surface was functionalized with 0.1 mL of 1 mM polyethylene glycol with thiol group (mPEG-SH) which was added to 3 mL of 5.4 nM GNRs and incubated for 48 hrs. To remove non-reacted mPEG-SH, the solution was centrifuged, decanted and redispersed in water several times. The GNRs thus prepared present an absorption peak of ~810nm (Figure 1b), and they have an aspect ratio of about 4.2, and a length of 50 nm (Figure 1c).

Photothermal- Optical Coherence Tomography

A PT-OCT imaging system was developed as illustrated in Figure 2a. The system used a conventional spectral domain OCT, which is similar to the one previously described.¹⁷ A superluminescent diode (SLD) with a central wavelength of 1310 nm and a bandwidth of 46nm was used as an OCT light source, providing an axial resolution of 13 μm in air. The light from the SLD was coupled into a fiber based Michelson interferometer, via an optical circulator. The light of the reference arm was reflected from a stationary mirror. A pump laser beam with an output power of ~ 25 mW and a wavelength of 808nm was used to photothermally excite the GNRs (absorption peak of ~ 810 nm). The pump laser was modulated at 50 Hz with a function generator.

The light from the SLD and the pump laser were combined in the sample arm with a dichroic mirror, and an XY pair of scanning mirrors was used to laterally scan the sample in a raster fashion. The light from the sample arm was focused into the sample with an achromatic objective lens with a focal length of 50 mm. The size of the focal spot on the sample was ~ 120 μm and ~ 20 μm for the 808 nm and 1310 nm light sources, respectively. A 2×2 optical fiber coupler was used to recombine the light backscattered from the sample and reflected from the reference mirror. The combined light was rerouted with the optical circulator towards a spectrometer. The spectrometer consisted of a 50 mm focal length collimator, a 1200-lines/mm transmitting grating, an achromatic lens with a 150 mm focal length, and a 14-bit, 1024 pixels InGaAs line scan camera with a maximum acquisition rate of 47 kHz. This spectrometer setup had a spectral resolution of 0.055 nm, which gave a maximum imaging depth of ~ 3.0 mm. A personal computer was used to synchronously control the acquisition of the camera and the square wave excitation of the pump laser. 1000-line M-mode acquisition was performed at each transverse position with an acquisition rate of 2238 Hz.

Figure 2b illustrates the PT-OCT data processing method which can identify the uptake of GNRs inside biological tissues. The GNRs absorb the energy from the 808nm light source, creating a change in the optical pathlength of the tissue, where the absorption is localized. Therefore, unlike scattering based imaging modality, photothermal contrast is directly related to optical absorption, leading to background free imaging of endogenous and exogenous contrasting agent (Figure 2c). The changes of optical pathlengths due to localized GNRs photothermal effect were detected by measuring the phase changes in the OCT signals using the PT-OCT system. The system sensitivity to measure the optical phase was evaluated at 120 dB,¹⁷ giving an ability to sense changes in optical pathlength as small as ~ 50 pm due to the GNRs absorption. The detailed data processing methods have previously been described in the literature, in terms of calculating the optical pathlength changes from the modulated optical phase signals¹⁷ and subsequently evaluated the concentration of absorptive substances within the scattering media.¹¹ Briefly, the OCT probe beam scanned the sample to obtain 3D data cube. At each spatial location, 1000 A scans were collected (M-scan). The conventional OCT algorithm was applied to the 3D dataset to obtain SLN morphological information. For photothermal imaging, the data processing was performed at each spatial location one by one. First, the phase information (i.e., the change of optical pathlength) was extracted from the complex OCT signals of the M-Scan at each spatial location. The strength of the tissue modulation due to the photothermal effect was then obtained by the Fourier transformation of the phase values, and converted into the concentration of SLN upon calibration (see Fig. 5c below).

Animal Preparation

36 mice (C57 BL/6) that weighed $\sim 23 \pm 2$ g, were used to image the uptake of GNRs within their left SLN *in situ*. All experimental animal procedures were in compliance with the

Federal guidelines for care and handling of small rodents. Each mice was anesthetized with vaporized isoflurane (0.2 L/min oxygen and 0.8 L/min air), and their body temperature was kept at 37 °C with the use of a heating pad. A volume of 100 μ L of GNRs (0.8 nM) was injected through the femoral vein of the right leg. At each time point (0, 0.25, 4, 8, 12, 24, 48, 96, 140, 320, 456 and 672 hrs) 3 mice were sacrificed and their left SLN was dissected and imaged with the PT-OCT system. During imaging, the SLN was kept in solidized 1% agar gel.

Results and Discussion

To demonstrate an application for using GNRs as a nanoprobe for high resolution imaging of SLN, we used two different optical imaging modalities, OCT and PT-OCT. Figure 3a shows the three-dimensional projection view of a dissected SLN at 48 hours after GNRs injection obtained with the OCT system. The inset in Figure 3d is a top view photograph of a dissected SLN, in which the areas of high GNRs uptake can be observed with a dark color. Figure 3b shows a cross-sectional cut of the volumetric SLN dataset at a depth of 240 μ m from the surface obtained with the OCT system, and it delineates the typical morphological features of the SLN. It can be observed, that the OCT image cannot detect the nanoparticle accumulation because of the low scattering contrast that is present on the GNRs. However, in the PT-OCT image (Figure 3c), the photothermal properties of the GNRs significantly enhance the imaging contrast. The PT-OCT image delineates the uptake of GNRs throughout the SLN, and by combining both the OCT and PT-OCT images we can distinguish the distribution of GNRs in several SLN structures. The intravenously injected GNRs migrate out of the venules and mostly accumulate in the superficial sinus, which is a channel that filters foreign organisms. Also, the GNRs accumulate at the cortex surrounding follicles, where mainly B cells and intrabecular sinus are filtered.

Using the PT-OCT system, it is possible to monitor the distribution of GNRs within the SLN structures with high resolution as a function of time and at defined depths. Figure 4 depicts several PT-OCT cross-sectional images of a lymph node at a depth of 120, 240, 360, 480, 600, 720 and 840 μ m below the surface, and at two time points (12 hrs and 96 hrs). At 12hr post-injection (Figure 4b), GNRs were present at qualitatively consistent levels. At 96 hrs (Figure 4c) the GNRs uptake by the SLN was significantly increased at all the depths.

Time dependent lymph node uptake of nanoparticles has been investigated by other researchers and typically focused on time points in the range of 6–120 hrs post-injection.^{18, 19} Also, the ability of surface modification of nanoparticles to enhance lymphatic drainage has been demonstrated.¹⁹ To further evaluate the kinetics of PEG modified GNRs uptake, PT-OCT images were acquired at 0, 0.25, 4, 8, 12, 24, 48, 96, 140, 320, 456 and 672 hrs after the 0.8 nM GNRs injection. This enabled us to visualize the time-dependent GNRs uptake within the SLN. Figure 5a presents projection images of the GNRs uptake within SLN at the specified time points. To quantify the GNRs uptake kinetics in SLN, the average concentration of GNRs within the SLN was calculated, by summing the PT-OCT signal strength (i.e. optical pathlength changes) across the whole SLN and then dividing it by the SLN volume. This value was compared with a calibration curve (Figure 5c) that was obtained using tissue phantoms made with known concentration of GNRs. Figure 5b presents the average concentration of GNRs within the SLN as a function of time. It is observed that the concentration reaches a peak of ~ 17 pM at 96 hours, and after that the concentration slowly declines. Compared to the initial GNRs injection (0.8 nM), the maximal lymph node uptake of about 2 % can be achieved with GNRs at 96 hr.

The thermal effect upon the pulsed laser irradiation is the physical base for PT-OCT to measure the GNRs accumulation within the SLN. However, the increase of temperature in

tissue is small. Based on a mathematical model developed by Adler *et al.*,¹¹ the estimated increase of temperature for a ~17 pM is ~0.1 °C, which is way below the threshold that would cause laser-induced tissue injury.

The use of GNRs enables us to understand the functionality of the SLN in many ways using PT-OCT. First, the GNRs are a source of high contrast that allows us to obtain high resolution images of different SLN structures. Second, the use of PT-OCT may be useful in delineating the migration pattern of the GNRs within different SLN structures as a function of time. And finally, the PT-OCT images allow the quantification of the circulation times of the GNRs. The miniaturized catheter has been developed for OCT to successfully image the interstitial tissue morphology *in vivo*.^{20, 21} Therefore, we expect that such miniaturized catheter can be integrated, in future, with the current PT-OCT probe so that *in vivo* imaging of GNRs uptake within the SLN would be feasible.

An ideal SLN contrast agent should be small enough to rapidly drain to the lymphatic tissue but large enough to stay within the lymphatic system during the procedure. The migration speed of certain contrast agents has been reported to be dependent on the shape, size (smaller sized nanoparticles migrate faster than larger sized nanoparticles) and surface composition.^{6, 22} The GNRs that were used for this study had an average length of 45 nm, and the surface was functionalized with PEG-SH to produce long blood circulation lifetimes and biocompatibility. PEG has well established stealth effect that can protect GNRs from extraneous matter by serum proteins and also it can reduce the clearance by reticuloendothelial system.²³ Also, PEG-modified GNRs shows lower cytotoxicity and improved *in vivo* circulation following intravenous injection in murine models.²⁴

Conclusion

We have used GNRs as a nanoprobe for enhancing 3D high resolution images of *in situ* SLN samples from a mice model. Compared to conventional scattering OCT, the PT-OCT system can monitor the distribution of GNRs within specific SLN structures through time. The uptake of GNRs within a SLN is slow (reaching a peak at ~96 hours) indicating that the GNRs have long circulation times. The resulting SLN images show that GNRs are suitable as a contrast agent. Although the imaging experiments were conducted *in situ*, we expect to be able to obtain these images *in vivo* and in real-time with the aid of a catheter. In the future, GNRs could also be used as a multifunctional probe for both diagnostic and therapeutic purposes.

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Figure 1.

(a) Scheme of preparation of gold nanorods as the nanoprobe. (b) VIS-NIR absorbance spectrum and (c) transmission electron microscopy image of the prepared GNRs.

Figure 2.

(a) Experimental setup of the PT-OCT system, where PC denotes the polarization controller. (b) Diagram of the data processing method used to image GNRs uptake in sentinel lymph node. (c) Schematic of GNRs-contrasting PT-OCT imaging. Unlike the scattering-based OCT, the PT-OCT imaging method can identify the uptake of GNRs in a highly scattering background because it is only sensitive to the absorptive GNRs.

Figure 3.

(a) Three-dimensional OCT projection image of a dissected SLN at 48 hours after GNRs injection. (b) 3D OCT view of SLN morphology with a cross-sectional cut at a depth of 240 μm below the top surface. (c) The same as in (b) but cross-sectional cut was from PT-OCT images to highlight SLN structures. (d) Schematic diagram and photograph of a dissected SLN. (Volume size= $2.5 \times 2.5 \times 2.0$ mm (x-y-z))

Figure 4.

Cross-sectional PT-OCT images was obtained at different depths (120, 240, 360, 480, 600, 720 and 840 μm) below the surface by slicing the 3D data cube [shown in (a)] acquired from the SLN at the time point of (b) 12 and (c) 96 hours after GNRs injection.

Figure 5.

(a) Uptake of GNRs within the sentinel lymph nodes at different time points. (b) Average concentration of GNRs throughout the whole SLN as a function of time. (c) Calibration curve obtained from experiments in tissue phantoms with known concentrations of GNRs. The linear curve maps the strength of the optical pathlength changes, which is detected by the PT-OCT system, with the concentration of GNRs.