Biomaterials 73 (2015) 142–148

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Dual-color photoacoustic lymph node imaging using nanoformulated naphthalocyanines

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ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form
30 August 2015
Accepted 9 September 2015
Available online 16 September 2015

Keywords:
Photoacoustic imaging
Optoacoustic
Organic nanoparticles
Naphthalocyanines
Sentinel lymph node mapping

ABSTRACT

Demarking lymph node networks is important for cancer staging in clinical practice. Here, we demonstrate *in vivo* dual-color photoacoustic lymphangiography using all-organic nanoformulated naphthalocyanines (referred to as nanonaps). Nanonap frozen micelles were self-assembled from two different naphthalocyanine dyes with near-infrared absorption at 707 nm or 860 nm. These allowed for noninvasive, nonionizing, high resolution photoacoustic identification of separate lymphatic drainage systems *in vivo*. With both types of nanonaps, rat lymph nodes buried deeply below an exogenously-placed 10 mm thick layer of chicken breast were clearly visualized *in vivo*. These results show the potential of multispectral photoacoustic imaging with nanonaps for detailed mapping of lymphatic drainage systems.

1. Introduction

The sentinel lymph node biopsy (SLNB) has become a standard clinical procedure as a less invasive preference to axillary lymph node dissection in breast cancer patients. In clinical practice, prior to SLNB, sentinel lymph nodes (SLNs) are identified intraoperatively to guide the surgical operations [1–3]. The typical clinical protocol includes: (1) injection of radioactive colloids (e.g., ^{99m}Tc), (2) rough identification of the SLN's position using a Geiger counter, (3) injection of a colored dye (e.g., methylene blue), (4) exposure of the SLN via visual identification, and (5) surgical removal of the SLN for histology [4,5]. From a safety point of view, conventional SLNBs use ionizing radiation and are not completely free from morbidity; associated complications such as seroma, lymphedema, nerve injury, and limitation of motion often occur [6,7]. More problematically, the low spatial resolution of the Geiger counter makes it impossible to noninvasively pinpoint the exact position of the SLN [8]. Therefore, noninvasive, nonionizing, and accurate imaging of SLNs is critical to relieve the burden of the cancer patients by providing possibilities to perform minimally invasive image-guided axillary staging using fine needles or noninvasively identify the metastatic SLNs using molecular targeting agents [9].

Recently, photoacoustic imaging (PAI) has been explored as a noninvasive lymph node mapping tool in both small animals and human breast cancer patients because it can sensitively visualize contrast enhanced SLNs in deep tissues (i.e., up to ~50 mm) with good ultrasonic spatial resolution (i.e., ~0.5 mm) [10–13]. Organic dyes such as methylene blue and indocyanine green have been typically used as PA lymph node tracers. However, these small molecules have concentration-dependent spectral properties and flow too quickly into successive lymph nodes from the sentinel node resulting in a high rate of false positives in axillary staging [14–16]. In addition, other inorganic contrast agents such as gold- and carbon-based nanostructures have been widely exploited as PA

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http://dx.doi.org/10.1016/j.biomaterials.2015.09.023
0142-9612/

lymph node agents in small animals due to strong localized surface plasmon resonance, biocompatibility, and efficient molecular targeting capability [17–21]. However, inorganic materials generally are associated with long-term safety concerns. Multispectral family of nanoparticles have been demonstrated with this approach, but not in the context of lymphatic mapping [22].

Conventional medical imaging tools typically use monochromatic energy sources. Thus, one contrast agent can be visualized at a single acquisition, and the acquired data is limited to a single spectrum. Optical imaging is uniquely able to provide multispectral parameters of multicolor contrast agents [23–26]. However, conventional planar fluorescence imaging or high resolution fluorescence microscopy suffers from either poor spatial resolution in deep tissues or shallow penetration, respectively.

In this study, we have used organic nanoformulated naphthalocyanines (referred to as nanonaps) for dual-color PA SLN mapping *in vivo*. The discovery of nanonaps was recently reported [27] and unique features include (1) exceptionally strong near-infrared (NIR) absorption in solution of greater than 1000 (additionally, these are nearly 100 times more absorbing than gold nanorods at the same mass concentration), (2) no potential heavy metal toxicity due to its organic nature, (3) multi-color imaging capability due to wide spectral tuning range in the NIR region, and (4) non-shifting spectral stability at ultrahigh optical densities. Nanonaps are formed from kinetically frozen Pluronic triblock copolymers which are formed with two biocompatible polyethylene blocks. Due to the hydrophobicity of the naphthalocyanines, excess surfactant can be removed at low temperatures through membranes while the dye is fully retained in the micelles. Here, we have noninvasively delineated two separate lymphatic systems and SLNs in small animals *in vivo* with combination of dual-color nanonaps and PAI. Further, the deep imaging penetration (~10 mm) in biological tissues was also achieved with aid of highly absorbing nanonaps *in vivo*. Therefore, we expect nanonaps to be beneficial in breast cancer staging as minimally invasive or noninvasive PA lymph node tracers.

2. Materials and methods

2.1. Nanonap formation

2 mg 5,9,14,18,23,27,32,36-octabutoxy, 2,3-naphthalocyanine (ONc) or 2,11,20,29,tetra-tert-butyl-2,3-naphthalocyanine (ZnBNc) was dissolved in 1 ml dichloromethane solvent, then the organic solution was added drop wise to 10 ml 10% (w/v) Pluronic F127 solution and was stirred until the dichloromethane evaporated. After centrifugation at 3500 g for 10 min, the supernatant were subjected to centrifugal filtration using Amicon Ultra-15 centrifugal filtration device with a 100,000 MWCO (Fisher #UFC9-100-24) at 4 °C until 200 μL of solution was retained in the filtration device. Water was then added back to the filtration device and the washing procedure was repeated in triplicate. Absorbance was measured with a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer).

2.2. Photoacoustic imaging system

A dark-field PAI system (Fig. S1) was utilized in all imaging studies [28]. We used a tunable OPO laser (Surelite OPO PLUS; Continuum) pumped by a Q-switch Nd:YAG laser (Surelite III-10; Continuum) to generate laser pulses with a pulse width of 4 ns and a repetition rate of 10 Hz. Optical wavelengths of 707 and 860 nm which match the optical absorption peaks of nanonaps were selected for *in vivo* and *ex vivo* PA imaging. After going through a spherical conical lens and hand-made optical condenser, the donut-shaped laser beam illuminated into targets. The measured laser pulse energy was approximately 1 mJ/cm^2, which satisfies with the safety limit of the American National Standards Institute (ANSI). To enhance acoustic coupling, we used a water bath having a bottom opening covered with a thin membrane. The generated PA signals were detected by a single-element spherically focused ultrasound transducer (V308; Olympus NDT) with a 5-MHz-center-frequency. The lateral and axial resolutions are 590 and 150 μm, respectively. With one laser pulse excitation at a fixed position, 1D depth-resolved PA images along the z axis were acquired, referred to as A-lines. By performing raster scanning along the x axis, 2D depth-sensitive PA images along the x and z axes were obtained. Additional raster scanning along the y axis allowed us to acquire the volumetric PA images. The amplified PA signals were recorded by an oscilloscope (MSO5204; Tektronix).

2.3. In vitro analyses of the PA sensitivity and spectrum

To measure the PA sensitivity and spectra of 707 (ZnBNc) and 860 (ONc) nm nanonaps, we prepared ten silicone tubes (508-001, Silastic laboratory tubing) filled with aqueous solutions of water and nanonaps with various concentrations. The silicone tubes were initially fixed onto an acrylic holder having a middle opening, and both distal ends of the tubes were glued using silicone sealant (RTV118, Momentive). Then, the silicon tubes were immersed in the water bath. To acquire the PA spectra, the excitation laser wavelength was tuned from 680 nm to 950 nm. Furthermore, the optical wavelengths of 707 nm and 860 nm were used to measure the PA sensitivities of two types of nanonaps.

2.4. In vivo and ex vivo PA imaging

We satisfied with the guidelines of Pohang University of Science and Technology (POSTECH) on the care and use of laboratory animals in all animal experiments. First, female Sprague Dawley rats (~250 g) were prepared for *in vivo* single-color PA lymph nodes mapping. A mixed solution of ketamine (85 mg/kg) and xylazine (15 mg/kg) was used to anesthetize the rats, and then a vaporized-isoflurane system was used to maintain the anesthesia during the *in vivo* experiments. Before PA imaging, the hair in the axillary regions was removed using depilatory gel. The PA images of the axially region were visualized before and after injection of each nanonaps (0.1 mL and 17.1 mg/mL) via the left forepaw pad. We have maintained the temperature of the rats using an electric heating pad. For *in vivo* dual-color lymph nodes mapping, female Balb/c hairless mice (~20 g) were prepared. After anesthetizing them, they were positioned on the sample stage, and then the dorsal regions were photoacoustically imaged. After acquiring the control PA image, 707 nm and 860 nm nanonaps (0.01 mL and 17.1 mg/mL) were injected simultaneously throughout the forepaw pads of both sides (i.e., left side; 707 nm nanonap and right side; 860 nm nanonap). After finishing all *in vivo* animal experiments, they were sacrificed using overdosed CO_2 gas, and the lymph nodes were extracted for *ex vivo* PA imaging.

3. Results

Nanonaps were generated as previously described [27]. A dichloromethane solution of naphthalocyanines (Nc) was added to an aqueous surfactant solution of Pluronic F127 and the organic solvent was evaporated, leading to the formation of nanonap frozen micelles. At 4 °C, free F127 micelles, but not nanonaps, were dissociated into small F127 unimers, allowing for removal of the free surfactant with low temperature centrifugal filtration. The frozen micelles were induced by the highly hydrophobic Nc dyes used, as shown in Fig. 1A. The 707 nm nanonaps were formed using