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Sentinel lymph node mapping by a near-infrared fluorescent heptamethine dye

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ABSTRACT

We describe a near-infrared fluorescent heptamethine dye (IR-780 iodide) with unique properties for sentinel lymph node (SLN) mapping in both small and large animals. This dye has a significant photo-brightening effect in serum and a long retention time in the lymphatic system which allows to acquire much higher signal-to-noise ratios. Injection of only 10 nmol of this dye permits SLNs to be imaged easily in pigs using excitation fluence rates of only 2 $\mu W/cm^2$. In addition, this dye has a unique stability property after formalin fixation in tissues which raises the possibility of developing new and sensitive means of detecting lymph nodes in harvested surgical specimens. This dye can be completely cleared from the circulation in a couple of days and does not cause acute systemic toxicity.

1. Introduction

Near-infrared (NIR) excitable fluorescent contrast agents hold great promise for non-invasive in vivo imaging due to low tissue autofluorescence and deep tissue penetration in this region of the electromagnetic spectrum [1,2]. Organic dyes and inorganic quantum dots (QDs) are two representative fluorescent contrast agents that are currently available in biomedical fields such as sentinel lymph nodes (SLN) mapping in both animal tests and clinical applications [3–6]. Inorganic QDs have unique optical properties, but their safety is still a serious concern since there are toxic elements in their cores [7,8]. Indocyanine green (ICG) is another popular NIR diagnostic reagent that is approved for clinical use in hepatic function examination and fluorescent angiography [9]. Recently, ICG fluorescence imaging has also been reported for SLN mapping in cancer therapy [6,10]. However, ICG is a water-soluble dye, and thus migrates into blood vessels as well as lymphatics and is rapidly washed out from the tissues which result in a short imaging time. By summarizing the current non-invasive imaging modalities, there is an urgent need to develop new imaging agent for future fluorescent optical imaging technologies. To develop an ideal NIR imaging agent for clinical application, at least the following general criteria should be considered: (a) the agent should have high fluorescence emitting and long retention time to acquire high signal-to-noise ratio; (b) the agent must be safe and not cause acute systemic toxicities to hosts or have long-term carcinogenetic potential in host tissues; (c) the agent need to be photostable to allow repeated imaging and emitting strong fluorescent signals above background levels and from deep tissues.

In this work, we have identified a heptamethine indocyanine dye IR-780 iodide as a prototypic NIR imaging agent with the properties mentioned above. The potential of SLN mapping guided by IR-780 iodide fluorescence imaging in both mice and pigs were determined.

2. Materials and methods

2.1. Reagents

The heptamethine cyanine dye IR-780 iodide (2-[2-[2-Chloro-3-[(1,3-dihydro-3,3-dimethyl-1-propyl-2H-indol-2-ylidene)ethylidene]-1-cyclohexen-1-yl]ethenyl]-3,3-dimethyl-1-propylindolium iodide) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Quantum dots with a 15–20 nm diameter was purchased from Invitrogen (Carlsbad, California, USA) and ICG was purchased from JiShi (Shenyang, Liaoning, China). Methylene blue (10 mg/ml) was purchased from Jumpcan (Taixing, Jiangsu, China) and 4-6-diamidino-2-phenylindole(DAPI) was from Zhongshan (Beijing, China).

2.2. Optical properties

To identify the optical properties of IR-780 iodide in methanol and serum, IR-780 iodide was incubated in methanol or 100% fetal calf serum (FSC) at 37 °C as the concentration of 1 μM. Photoluminescence were determined using a Cary Eclipse

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Fluorometer (Varian Instruments, Palo Alto, CA) (740 nm excitation, continuous wavelength from 750 to 900 emission) and Kodak In-Vivo FX Professional Imaging System. As a contrast, ICG (1 μM) was also examined in both methanol and FSC. For serum stability test, IR-780 iodide were filtered through a 0.22-μm filter and then was incubated in 100% FCS at 37 °C as the concentration of 1 μM for 1 h ($n = 6$). Fluorescence intensities were detected by Cary Eclipse spectrofluorometer (740 nm excitation, 802 nm emission) every 10 min.

2.3. NIR imaging

The animals or dissected tissue/organs imaging was performed using a Kodak In-Vivo FX Professional Imaging System (New Haven, CT) equipped with fluorescent filter sets (excitation/emission, 770/830 nm). The field of view was 10 cm in diameter. The frequency rate for NIR excitation light was 2 μW/cm^2. The camera settings included maximum gain, 2 × 2 binning, 1024 × 1024 pixel resolution, and an exposure time of 20 s. Mice were anesthetized with pentobarbital and maintained in an anesthetized state during imaging procedure.

2.4. Small and large animal studies

Animal protocols were in accordance with the Animal Care and Use Committee Guidelines of the Third Military Medical University. Mice of 20–25 g were anesthetized with pentobarbital intraperitoneally. Mice were shaved, and lay on the pallet of Kodak In-Vivo FX Professional Imaging System. IR-780 iodide and ICG of 0.5 nmol, QDs of 10 pmol [3] in water were injected intradermally into mouse paws, and imaged by KODAK In-Vivo imaging system. Methylene blue was then reinjected at the same site to confirm the nodes with the NIR image.

For large animal studies, pigs were anesthetized with pentobarbital by intravenous injection. IR-780 iodide of 10 nmol in water was injected intradermally in mammary tissue. After the injection of NIR tracer, 200 μl of 1% methylene blue was injected into the same site to identify the SLN with the NIR image. The lymph nodes were then removed and evaluated for NIR imaging.

After imaging, the nodes were fixed by 4% formaldehyde and imaged. For histological study, nodal tissue was embedded in optimal cutting temperature compound (OCT) (Zsbio, Beijing, China), snap-frozen, and cryo-sectioned at 6 μm. Sections were stained with hematoxylin and eosin (H + E) or DAPI (Zsbio, Beijing, China), and then sections were examined by Light microscope and laser scanning confocal microscopy.

2.5. Acute toxicity study

To determine the acute toxicity of IR-780 iodide in small animal, 6 immunocompetent wild type mice were intravenous injected with 100 nmol of IR-780 iodide in a total volume of 100 μl in normal saline. The control mice were intravenously injected with the 100 μl of saline ($n = 6$). The body weight and physical activities were observed within 7 days. At the seventh day after IR-780 iodide administration, all mice were sacrificed and the blood samples were examined for biochemical indicators and blood cells count. All tissues were fixed in 4% formaldehyde, made into paraffin sections and stained with hematoxylin and eosin for histological examination.

2.6. Statistical analysis

Data are presented as mean ± standard error. Data were analyzed using Student *t*-test, a *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Optical properties and stability of IR-780 iodide

IR-780 iodide is a lipophilic cation with peak absorption at 780-nm and molecular weight of 667 Da which can be detected conveniently by a NIR fluorescent detection system. A rigid cyclohexenyl ring in the heptamethine chain with a central chlorine atom maintains photostability, increases quantum yield, decreases photobleaching, and reduces dye aggregation in solution (Fig. 1a). The fluorescence emitting of IR-780 iodide in methanol was slightly higher than ICG. However, it was greatly enhanced in serum and this enhancement effect was not achieved in ICG (Fig. 1b and c). After incubation in 100% FCS at 37 °C for 1 h, the total fluorescence emission of IR-780 iodide showed a slightly increase in first 20mins and then maintained at a stable level which indicated that IR-780 iodide was pretty stable in serum (Fig. 1d).

3.2. SLN mapping in mice

In all mice, SLNs were identified as early as 5 min after the injection of the paws ($n = 6$) with IR-780 iodide using NIR fluorescence image system. The lymph nodes and lymphatic vessels were visualized within a few minutes and lasted for more than 3 h, and then gradually decreased. Fig. 2a showed a photograph of the imaging region of a mouse with hair removal before NIR imaging and the consecutive NIR images at 5 min, 1, 2 and 3 h post-injection of IR-780 iodide, respectively.

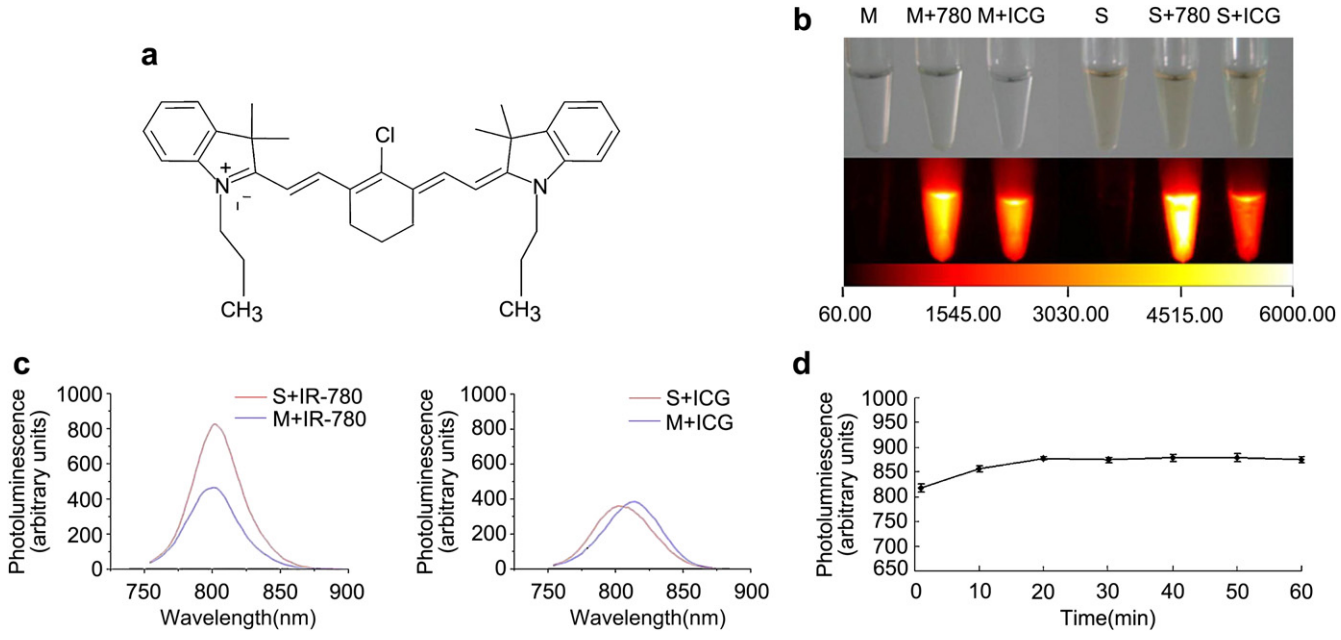

Fig. 1. Physical and optical properties of IR-780 iodide. (a) Chemical structure of IR-780 iodide. (b) Color and fluorescent image of IR-780 iodide and ICG in methanol (M) and 100% FSC (S) at the concentration of 1 μM and imaged immediately post-preparation. (b) Photoluminescence intensity of IR-780 iodide and ICG in methanol and 100% FCS. (d) Fluorescence stability of 1 μM IR-780 iodide, in 100% FCS, at 37 °C for 1 h. Each valid point and vertical bar shows mean ± SD.