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Dextran-based fluorescent nanoprobes for sentinel lymph node mapping

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ABSTRACT

Biopsy of sentinel lymph node (SLN) has become a common practice to predict whether tumor metastasis has occurred, so proper SLN positioning tracers are highly required. Due to many drawbacks of SLN tracers currently used, developing ideal, biosafe SLN imaging agents is always an urgent issue. The current study designed a novel fluorescent nanoprobe for accurate SLN mapping. Dextran-based nanogel (DNG) was prepared through a highly efficient self-assembly assisted approach and serves as a multifunctional platform for conjugating wide spectra emitting fluorescent agents. The newly fabricated fluorescent DNG (FDNG) could be designed with optimum size and stable fluorescent intensity for specific SLN imaging. Furthermore, a long-term dynamic course *in vivo* (from 1 min to 72 h) revealed the satisfactory specificity, sensitivity, and stability for SLN mapping. Most importantly, both *in vitro* and *in vivo* evaluations indicated that FDNG had fine biosafety and biocompatibility with lymphatic endothelial cells. All these results supported that FDNG could be used as highly efficient molecular imaging probes for specific, sensitive, stable, non-invasive, and safe SLN mapping, which provides efficient and accurate location for SLN biopsy and thus predicts tumor metastasis as well as directs therapies. Besides, our recent studies further demonstrated that DNG could also serve as a specific and controllable drug carrier, indicating a potential application for specific therapies of various lymph-associated diseases.

1. Introduction

Sentinel lymph nodes (SLNs) are the first lymph nodes (LNs) that are reached by cancer cells when primary tumor metastasis occurs *via* the lymphatic vessels (LVs) [1–3]. It is generally believed that if no metastasis is observed in the SLN, the risk of metastasis extending to the regional LNs will be low [4]. Therefore, the accurate localization of SLNs has been considered a very important step towards the biopsy of SLNs to determine whether tumor metastasis has occurred [5]. At present, SLNs are usually probed with methylene blue or with isotopes [6], but all these methods have drawbacks. For example, methylene blue displays non-selective *in vivo* distribution and a large chance that the second or even third stand LNs may be mistaken for SLNs, which may result in incorrect conclusions about cancer metastasis states. Additionally, methylene blue guided methods need invasive surgery and thus the detection of SLNs largely depends on the operator's experience. Radionuclide lymphatic imaging involves complex preoperative preparation procedures and expensive equipment, and the radioactivity also limits the application of isotopic probes. The demand for specific, biosafe, and stable nanoprobes that can provide accurate and long-lasting mapping of SLNs has become an overwhelming issue in biomedical field.

The rapid development of molecular imaging methods offers great possibilities for highly efficient and non-invasive imaging of SLNs [5,7–12]. In various strategies employed for SLN detection [8,13–16], fluorescent imaging has proven to be an efficient approach to address this challenge because of its high sensitivity and radiation-free nature [17]. Among various fluorescent agents, the inorganic nanocrystals [18] including quantum dots (QDs)

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[19–22] and fluorescent nanodiamonds [23] have been reported as probes for SLN mapping. Nevertheless, the potential toxicity of QDs and the complicated fabrication procedure of nanodiamonds greatly compromise their usage for *in vivo* imaging. Meanwhile, as a class of nanocarriers possessing three-dimensional hydrophilic polymer networks [24,25], nanogels have been favored in many biomedical applications, including drug delivery and cell imaging, *etc*. [26–31]. Noh and his co-workers synthesized a near-infrared (NIR) fluorescent probe that consisted of pullulan-cholesterol nanogels and IRDye800 payload [32]. They found that these nanogels, with a hydrodynamic diameter of 30–50 nm, could accumulate selectively in the LNs and thus were very efficient for specific SLN mapping.

Although some existing reports have preliminarily described the potential applications of polymeric fluorescent nanogels for SLN mapping [31–33], several important issues still need to be addressed to predict practical clinical feasibility of fluorescent nanogels for specific and safe SLN mapping: 1) The feasibility of fluorescent nanogel fabrication based on biomacromolecule with proper diameters and stable fluorescence properties; 2) The long-term *in vivo* dynamic course of the fluorescent nanogels for SLN mapping; 3) The biosafety of these fluorescent nanogels; and 4) The possible impact of fluorescent nanogels on the characteristic and function of lymphatic endothelial cells (LECs), a dominant cell type that lines the inner surface of LVs.

To address the above issues, in the current study, we designed a dextran-poly (acrylic acid) nanogel (DNG) through a highly efficient self-assembly assisted (SAA) approach referred to our previous study [34], and then conjugated it with amine-bearing fluorochromes with broad-spectrum fluorescence to produce various fluorescent nanogels (FDNGs). The characteristic of a representative green light emitting FDNG as a probe for SLN imaging was investigated in detail. Cytotoxicity, biocompatibility, and FDNG's influence on cell function were further evaluated to predict the biosafety in cell level. Most importantly, FDNG was introduced for SLN mapping in a BALB/c mouse model and an *in vivo* long-term dynamic course was further investigated to explore the feasibility for accurate SLN location. Besides, FDNG's influence on body weight, behavior, blood biochemistry, and important organs was also evaluated to predict the biosafety for future clinical application.

2. Materials and methods

2.1. Materials

Dextran-poly(acrylic acid) nanogel (DNG) was fabricated *via* an SAA method developed by our group, as described in previous reports [34,35]. 5-Aminofluorescein (5-AF) was purchased from Sigma Aldrich (St. Louis, U.S.A.), 7-amino-4-methyl coumarin (AMC) was purchased from Aladdin (Shanghai, China), near-infrared light emitting CdSeTe/CdS/ZnS core/shell/shell quantum dots (QDs) were fabricated in our laboratory as described in previous works [36,37] and further modified with glutathione (GSH) [38]. 1,1′-dioctadecyl-3,3,3,3′-tetramethyl-indocarbocyanine-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) was purchased from Molecular Probes (Oregon, U.S.A.), and matrigel was from BD Biosciences (New Jersey, U.S.A.). Microvascular Endothelial Cell Growth Medium-2 (EGM2-MV) was purchased from Lonza (Basel, Switzerland).

2.2. Preparation and characterization of FDNGs

FDNGs were fabricated through conjugation of DNG with three different amine-bearing fluorescent agents, 5-AF, AMC, or GSH-modified CdSeTe/CdS/ZnS core/shell/shell QDs respectively, under 1- (3-dimethylaminopropyl) -3- ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) catalysis to produce green, blue, or deep-red fluorescence-emitting nanogels. The fluorescence intensities exhibited by FDNGs were measured using a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Japan), with excitation wavelengths of 350 nm, 492 nm, and 450 nm for FDNG(AMC), FDNG(5-AF), and FDNG(QDs) samples respectively. As FDNG(5-AF) showed strong green fluorescence and green light was readily detected by both human eyes and instruments [39], FDNG(5-AF) was used for future studies. The diameters and zeta potentials of DNG and FDNG(5-AF) were characterized using Zetasizer Nano ZS90 (Malvern, U.K.). TEM images were recorded using a JEM-2100 (JEOL, Japan) TEM system, and the samples were negatively stained with phosphotungstic acid. ^{1}H NMR spectra of DNG and FDNG(5-AF) samples (dissolved in D_2O, adjusted by 40 wt% NaOD) were measured using an Avance III 400 MHz NMR spectrometer (Bruker, Switzerland). The fluorescence stability of FDNG(5-AF) sample was tested during a storage period of over 30 days at 37 °C.

2.3. In vivo lymphatic node mapping

Female BALB/c mice aged 5–6 weeks (acquired from the Animal Institute of Chinese Academy of Science, Shanghai, China) were maintained under specific pathogen free conditions. All experiments employing mice were performed in accordance with the Chinese NIH guidelines for the care and use of laboratory research animals.

For *in vivo* experiments, according to Young-Woock Noh's methods [32], the FDNG(5-AF) nanoprobe (20 μg of samples in 20 μL of water) was intradermally injected into the front paw of a mouse and imaged for *in vivo* migration of FDNG(5-AF) nanoprobe using a fluorescent optical imaging system. The SLN and LVs of the mice injected with FDNG(5-AF) nanoprobe were photoed at 0, 1 min, 5 min, 30 min, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h. Methylene blue dye at a concentration of 1% was then reinjected at the same site to further confirm the position of SLN. After skin removal, photographs of SLN were obtained using a Canon digital camera.

2.4. In situ histofluorescence

The *in situ* distribution of FDNG(5-AF) was analyzed by dissecting the axillary SLN after 12 h of injection. According to Noh's methods [33], the lymph node was embedded, frozen, cut to sections, and transferred to glass slides. The slides were stained with rabbit anti-mouse Lymphatic Vessel Endothelial Hyaluronic Acid Receptor 1 (LYVE-1) (Abcam, U.S.A.) to label lymphatic endothelial cells (LECs) as described in our previous study [40].

2.5. In vitro co-incubation of LECs and FDNG(5-AF)

To further analyze the possible mechanism of FDNG(5-AF) on SLN mapping at cell level, *in vitro* experiments were performed by incubating FDNG(5-AF) with LECs, a dominant cell type that lines the inner surface of LVs in lymph node. The isolation, culture, and identification of LECs were described in our previous study [40]. FDNG(5-AF) was added into EGM-2-MV to a final concentration of 100 μg/mL, and co-incubated with 0.5×10^6 LECs for 24 h. The resultant LECs were abbreviated as FDNG-LECs. Fluorescence images were captured under a fluorescence microscope (IX-70, Olympus, Japan) after washing and fixing. The positive rate of FDNG(5-AF) uptake was analyzed *via* a flow cytometer (Beckman Coulter, U.S.A.). For TEM observation, samples were prepared according to previous reports [41] and observed with TEM (Quanta 200, FEI Hillsboro, U.S.A.).

2.6. In vitro cytotoxicity and proliferation assays

Cytotoxicity of FDNG(5-AF) on LECs, kidney NRK cells, and liver BRL-3A cells were evaluated by MTT method (NRK and BRL-3A cell were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China). Cells were plated at a density of 6×10^3 cells/well in 96-well plates and incubated with FDNG(5-AF) at different concentrations for 24 h or 48 h followed by MTT assays. For cell proliferation evaluation, LECs or FDNG-LECs were seeded into 96-well plates at a density of 2×10^3 cells/well, and cell proliferation rates were detected daily for eight days by MTT method. Data expressed as the mean ± the standard deviation was used to prepare the growth curves.

2.7. Cell functional evaluation

FDNG-LECs in EGM-2-MV were seeded onto matrigel-coated 24-well plate at a density of 2×10^4 cells/well and then incubated for at least 4 h at 37 °C. The formation of microtube-like structure was analyzed using a Nikon TS-100 microscope and images were captured with a Nikon digital camera (DXM 1200). Uptake of DiI-Ac-LDL by FDNG-LECs was analyzed *via* fluorescence microscopy using a previously described method [42].

2.8. In vivo biosafety evaluation

BALB/c mice were randomly injected with 20 μL saline (control group) or 20 μL FDNG(5-AF) (1 mg/mL). At predetermined time points, mice were weighed and assessed for behavior changes. Using a standard blood collection technique from the heart, blood was drawn for biochemistry examination. For blood analysis, 1 mL of blood was collected from mice and separated by centrifugation into cellular and plasma fractions. Upon the completion of the last time point, mice were sacrificed by isoflurane anesthetic. Major organs in both FDNG(5-AF) treated mice and control mice were harvested, fixed in 4% neutral buffered formalin, embedded routinely into paraffin, stained with hematoxylin and eosin (H&E), and the histopathological lesions were examined and evaluated according to previously reported methods [43].

2.9. Doxorubicin (DOX) loading

DOX was conjugated onto DNG *via* hydrazone bond as described in previous report with minor modifications [44]. The hydrodynamic diameter ($<D_h>$) of the resultant DNG(DOX) was acquired from Zetasizer Nano ZS90 (Malvern, U.K.), while