Published in final edited form as:

J Drug Target. 2018 ; 26(5-6): 494–504. doi:10.1080/1061186X.2018.1433681.

Indocyanine green nanoparticles undergo selective lymphatic uptake, distribution and retention and enable detailed mapping of lymph vessels, nodes and abnormalities

John C. Kraft^a, Piper M. Treuting^b, and Rodney J. Y. Ho^{a,c}

¹Department of Pharmaceutics, University of Washington, Seattle, WA, USA

^bDepartment of Comparative Medicine, University of Washington, Seattle, WA, USA

^cDepartment of Bioengineering, University of Washington, Seattle, WA, USA

Abstract

The distributed network of lymph vessels and nodes in the body, with its complex architecture and physiology, presents a major challenge for whole-body lymphatic-targeted drug delivery. To gather physiological and pathological information of the lymphatics, near-infrared (NIR) fluorescence imaging of NIR fluorophores is used in clinical practice due to its tissue-penetrating optical radiation (700–900 nm) that safely provides real-time high-resolution *in vivo* images. However, indocyanine green (ICG), a common clinical NIR fluorophore, is unstable in aqueous environments and under light exposure, and its poor lymphatic distribution and retention limits its use as a NIR lymphatic tracer. To address this, we investigated in mice the distribution pathways of a novel nanoparticle formulation that stabilises ICG and is optimised for lymphatic drug delivery. From the subcutaneous space, ICG particles provided selective lymphatic uptake, lymph vessel and node retention, and extensive first-pass lymphatic distribution of ICG, enabling 0.2 mm and 5–10 cell resolution of lymph vessels, and high signal-to-background ratios for lymphatic vessel and node networks. Soluble (free) ICG readily dissipated from lymph vessels local to the injection site and absorbed into the blood. These unique characteristics of ICG particles could enable mechanistic studies of the lymphatics and diagnosis of lymphatic abnormalities.

Keywords

Lymphatic drug delivery; lymphatic fluorescence imaging; small molecules; nanoparticles; indocyanine green; near-infrared fluorescence imaging

Introduction

The lymphatic vasculature is a distributed, whole-body network of vessels and nodes that converges toward the thoracic duct, which runs ventrally up the spine in the thoracic cavity. While blood capillaries deliver oxygen and nutrients to cells *via* the interstitial space, most

CONTACT Rodney J. Y. Ho, rodneyho@uw.edu, University of Washington, Box 357610, Seattle, WA 98195-357610, USA.

Disclosure statement

The authors report no declarations of interest.

of the interstitial fluid and its solutes are drained by lymphatic capillaries rather than being reabsorbed into the blood through venous capillaries [1]. As a whole, the lymphatic system is central to fluid homeostasis, immune function, fat absorption in the gut, reverse cholesterol transport and disease [2]. Blockages of lymph drainage and flow (e.g. due to surgical interventions such as mastectomy) can lead to peripheral oedema (lymphedema), which afflicts 4% of the world's population [3]. Moreover, cancer and microbes exploit and block lymphatic pathways [4], and HIV infects cells of lymph nodes (LNs) throughout the body [5]. Despite the prevalence of occluded lymph flow and the lymphatic system being central to devastating diseases, there exists a gap in our ability to safely and efficiently visualise in detail and deliver drug to the extensive and distributed network of lymph vessels and nodes in the body. This is largely because current imaging and drug targeting agents do not widely distribute through and are not sufficiently retained within lymph vessels, nodes and overall lymphatic networks.

It is now becoming appreciated that small molecules in the systemic blood circulation have difficulty in penetrating into and being retained in lymph nodes throughout the body. As a result, subcutaneous (SC) injection as opposed to intravenous (IV) (or oral (PO)) dosing has been leveraged to transiently increase small molecule drug levels in lymph nodes [6], however, these elevated drug levels rarely persist for extended durations. In 2003, we first hypothesised and verified [7], and others recently confirmed in prospective clinical studies [8–10], that orally dosed antiretroviral small molecule drug combinations fail to maintain sufficient lymph node levels [11]. To overcome this insufficiency of small drug molecules in lymph nodes, our laboratory has developed a novel nanoparticle platform that enables targeted localisation of drug combinations in lymph nodes. We found that multiple drugs formulated in this nanoparticle platform are not only capable of distributing widely to lymph nodes throughout the body, but also provide exposure in cells of lymph nodes and the blood for an extended time. Thus, long-acting delivery of small molecule drug combinations (containing three or more hydrophilic and hydrophobic drugs) to lymph nodes throughout the body is feasible [11–13].

Recently, we leveraged this nanoparticle platform with its unique formulation and preparation methods to stabilise the near-infrared (NIR) fluorophore indocyanine green (ICG) [14]. NIR fluorescence imaging using non-ionizing 700–900nm optical radiation affords real-time *in vivo* imaging with high resolution and tissue penetration due to reduced photon scattering, light absorption and autofluorescence of biological tissues [15], and is an emerging technology in clinical practice [16]. The unique ICG particles we developed maximally boost the ICG fluorescence emission intensity [14] with a formulation optimised for widespread lymphatic distribution following subcutaneous (SC) administration [11,17]. The *in vivo* stability of these particles minimises the rapid release of free ICG, which reduces the likelihood of potential off-target toxicities. Moreover, reduction of free ICG improves the signal-to-noise ratio as the presence of free ICG, which binds non-specifically to interstitial and tissue proteins, contributes to background fluorescence signal. Injectable NIR protein- and nanoparticle-based lymphatic tracers have been reported [18,19], and free ICG is commonly used off-label in the clinic for lymphatic visualisation and sentinel lymph node dissection [20]. However, in preclinical models [18,19] and humans [21], these agents, including those reported for nanoparticle and liposome formulations, dissipate quickly from

lymph vessels and nodes near the injection site and do not provide sufficient detail of lymph vessel and node networks [22]. Moreover, studies show radiotracers such as subcutaneously administered ^{99m}Tc and blue dyes readily diffuse out of lymph vessels and deposit into nearby tissues or enter the blood [23,24]. As a result, blue dye tracers may leave tattoos for months, and furthermore, they carry a risk of skin necrosis and anaphylaxis [25].

We hypothesised that these limitations of current lymphatic imaging agents may be largely overcome if our novel ICG particles enabled widespread lymphatic distribution and retention of ICG specifically within lymph vessels and nodes. In this report, we show that following subcutaneous administration in mice, ICG particles provided selective lymphatic uptake, lymph vessel and node retention, and extensive first-pass lymphatic distribution of ICG, enabling 0.2 mm and 5–10 cell resolution of lymph vessels, and high signal-to-background ratios for lymphatic vessel and node networks. Soluble (free) ICG readily dissipated from lymph vessels local to the injection site and absorbed into the blood. Due to the widespread lymphatic distribution and retention of ICG particles, coupled with the maximally enhanced ICG fluorescence quantum yield, the safety of ICG and the nanoparticle formulation, as well as the convenience and cost-effectiveness of NIR imaging, these novel ICG particles are likely a useful tool for assessing lymphatic anatomy and function.

Materials and methods

Reagents

Phospholipids 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-methoxypolyethylene glycol-2000 (DSPE-mPEG₂₀₀₀) were purchased from Avanti Polar Lipids (Alabaster, AL). Indocyanine green (ICG, $\text{C}_{43}\text{H}_{47}\text{N}_2\text{NaO}_6\text{S}_2$, sodium 2-[7-[3,3-dimethyl-1-(4-sulfonatobutyl)-benz[e]indolin-2-ylidene]hepta-1,3,5-trien-1-yl]-3,3-dimethyl-1-(4-sulfonatobutyl)benz) was purchased from Sigma-Aldrich (St. Louis, MO). Qtracker 800 non-targeted quantum dots (PEGylated, ~20 nm diameter) were purchased from Invitrogen (Q21071MP, Molecular Probes; Eugene, OR). Other reagents were analytical grade or higher.

Optical imaging

Methods described previously were used to prepare ~60 nm ICG nanoparticles (ICG particles) where ICG molecules are complexed and stabilised by lipid excipients DSPC and DSPE-mPEG₂₀₀₀ [14]. Free ICG was prepared in saline with 8% (v/v) DMSO. All animal procedures complied with and were approved by the University of Washington Institutional Animal Care and Use Committee. Male and female 2–24-month old C57BL/6 mice were used, originally obtained from Charles River Laboratories (Wilmington, MA). Mice were kept under pathogen-free conditions, exposed to a 12 h light/dark cycle, and received food *ad libitum* prior to imaging. Mice were anaesthetised with 1.5% isoflurane, shaved to remove fur and placed on a 37 °C platform in an IVIS Lumina II (Caliper Life Sciences, PerkinElmer; Waltham, MA). IVIS imaging settings were: fluorescence, 3 s exposure (except 0.5 s exposure for Figure 1 data only), small binning, 2F/stop, Ex/Em 745/ICG (for ICG) and 570/ICG (for QD800). A 25–40 μL bolus of ICG particles or free ICG in buffered saline or 25 μL of QD800 in PBS was injected subcutaneously (SC) into the top (dorsum) of

hind feet to avoid the medial tarsal and lateral marginal blood vessels (0.5 nmol ICG/foot; 5 pmol QD800/foot). QD800 dose is equivalent to ICG particle dose at ~100 ICG molecules/particle. For the plasma pharmacokinetic study, mice were injected SC in the inner hind left leg about 0.5 cm above the ankle with 400 μ M ICG solutions of free ICG or ICG particles at a dose of 3.0 mg/kg. Whole blood was collected *via* cardiac puncture at terminal time points (2.5 and 5 min), placed into K2 EDTA (7.2 mg) vacutainer tubes, before isolating plasma by centrifuging at 2348*g* (5000 rpm) for 10 min and storing at -80°C . Room temperature whole plasma samples were measured for ICG fluorescence intensity using a fluorescence plate reader [Victor3 V 1420-040 Multilabel Plate Reader (PerkinElmer, Waltham, MA) with a tungsten-halogen continuous wave lamp (75 W, spectral range of 320–800 nm) and excitation (769 ± 41 nm) and emission (832 ± 37 nm) filters (Semrock, Rochester, NY) using 100 μ L of sample in flat bottom, untreated 96-well plates (Grenier Bio-one, Monroe, NC)]. Plasma ICG fluorescence intensity was converted into plasma ICG concentration (ng/mL) using standard curves of blank neat plasma spiked with fresh free ICG or ICG particles. All mice tolerated the injections well and were monitored for respiratory rate and heart rate. No changes related to the injections were observed. Experiments were performed with at least three independent replicates (specified in figure legends). Data collection methods were predetermined for all experiments, and animals were assigned randomly to treatment groups. No outliers were excluded. For region of interest (ROI) analysis of the foot injection site (Figure 1(B)) and of lymph nodes and the thoracic lymph duct (Figure 3(C–E)), 0.401 and 0.441 cm^2 circles were used, respectively, to collect total efficiency (a.u.) values using Living Image 4.5 (Caliper Life Sciences, PerkinElmer; Waltham, MA).

Immunohistochemistry

Podoplanin—The University of Washington's Histology and Imaging Core performed the immunohistochemistry optimisation and staining for podoplanin, using syrian hamster polyclonal anti-mouse podoplanin (1:250 dilution; BioLegend, catalogue number 127402). We used 5 μ m thick formalin-fixed paraffin-embedded sections for the staining. First, the slides were baked for 30 min at 60°C and deparaffinized on the Leica Bond Automated Immunostainer (Leica Microsystems, Buffalo Grove, IL). Antigen retrieval was performed with citrate buffer, pH 6.0, at 100°C for 20 min. All subsequent steps were performed at room temperature. Blocking consisted of Leica peroxide block for 5 min. Additional blocking was performed with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, catalogue number 017-000-121) in tris-buffered saline (TBS) for 20 min. The primary antibody, podoplanin, was diluted in Leica Primary Antibody Diluent and was applied for 30 min. A peroxidase-conjugated secondary antibody, goat anti-syrian hamster IgG (1:500 dilution; Jackson ImmunoResearch Laboratories, Inc., catalogue number 107-035-142) in 5% normal donkey serum and TBS for 30 min was applied. Antibody complexes were visualised using Leica Bond Mixed Refine (DAB, 3,3'-diaminobenzidine) detection $2 \times$ for 10 min. Tissues were counterstained with haematoxylin for 4 min followed by two rinses in water and dehydrated through graded alcohol to xylene. Slides were mounted with a synthetic mounting media. Slides were imaged using a Nikon Eclipse 90i automated microscope and a $20 \times$ objective lens. Unless otherwise specified all reagents were obtained from Leica Microsystems.

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). All statistical comparisons were performed with the two-tailed *t*-tests with significance probability α set at .05.

Results

Small molecule versus nanoparticle uptake and distribution

In a preliminary study, we observed soluble ICG (free ICG) in the SC space readily distributed equally well into lymph and blood vessels while ICG bound to novel nanoparticles (ICG particles) preferentially distributed to lymph but not blood vessels. Unlike free ICG, which readily redistributed to adjacent tissues and blood capillaries, the fraction of ICG particles in lymph vessels was retained without leaking into neighbouring tissues. Thus, to characterise the differential uptake and distribution of free ICG versus ICG particles, equal doses of free ICG and lipid-bound ICG were injected SC in the dorsal foot of mice and NIR fluorescence imaging over the skin was performed at 5, 30 and 60 min. As a control, we compared these results with quantum dot nanoparticles (QDs) that emit NIR wavelengths similar to those of ICG and have the same PEGylated exposed surface as ICG particles (Figure 1(A, B)). Rapid clearance of ICG particles from the SC site is detectable as a steep rate of decline in NIR intensity, while NIR intensity increased in mice treated with free ICG ($k = -2.07$ vs. $+1.41 \times 10^{-2} \text{min}^{-1}$) (Figure 1(B)). This increased NIR intensity for free ICG is due to non-specific protein binding of soluble ICG in the interstitial space and nearby tissues [26], instead of draining into blood or lymph vessels. In mice treated with the smaller QDs (~20 nm vs. ~60 nm ICG particles), QDs cleared from the SC injection sites at a much slower rate ($k = -1.01 \times 10^{-2} \text{min}^{-1}$) than ICG particles (Figure 1(B)), verifying the unique property of these ICG particles to rapidly clear from the SC space and distribute into lymph vessels.

Differential lymphatic and blood distribution of free ICG versus ICG particles

We then followed the movement of free ICG and ICG particles from the SC foot injection site to the nearby tissue in the mouse hind leg. To differentiate the distribution of free versus nanoparticle-bound ICG into blood, adjacent tissue and lymph, mice were dosed SC with free ICG or ICG particles in the dorsal foot and detailed ICG distribution was analysed. ICG appeared in the blood (saphenous vein) and nearby tissue more extensively in mice treated with free ICG (Figure 2(A–C, G)) than with ICG particles, which remained localised to the popliteal LN (Figure 2(D–F, H, I)). Mice with ICG particles in their popliteal LN did not have ICG in the nearby saphenous vein (Figure 2(D–F, H)) and the vessel connecting the popliteal and sciatic LNs was clearly delineated (Figure 2(I)). To further verify the extent that free ICG and ICG particles enter the blood from the SC space, we measured the plasma ICG concentration in mice following a 3.0 mg/kg dose of ICG given as free ICG or ICG particles, soon after drug administration. As shown in Table 1, ICG concentrations were significantly higher in plasma at early time points following free ICG dosing than after dosing ICG particles. These data suggest that ICG particles given SC remain in and distribute *via* the lymphatics (vessels and nodes) with minimal dissociation (or release of ICG from particles) without entering blood vessels as free ICG; whereas free ICG can

readily enter the blood circulation from the SC space and may leak out of the lymphatics and enter nearby tissues and blood. Such movement of ICG solute in and out of lymph vessels would greatly diminish the signal resolution (due to increased background fluorescence in nearby tissue) and the distribution of ICG throughout the lymphatics, which in turn would reduce the ability to target ICG specifically to the extensive network of lymph vessels and nodes.

Extensive distribution and retention of ICG particles in the lymphatic system

To visualise the whole-body lymphatic distribution of free ICG and ICG particles after a single SC dose in mice, we systematically analysed the ICG fluorescence intensity in the mouse lymphatic network that drains the right hind foot at 1, 5 and 30 min following SC injection. As shown in Figure 3(A), free ICG was visualised only in the lower part of the mouse lymphatic system (popliteal LN, sciatic LN, medial iliac LN and lumbar LN) at 30 min, whereas with ICG particles, this lower lymphatic portion was clearly visualised at 1 min post-injection, and the thoracic duct was clearly visualised at 5 min post-injection (Figure 3(B)). At 30 min post-injection, the signal from ICG particles detailing the lymphatic network did not readily fade from the signal previously observed at 5 min (Figure 3(B)), suggesting ICG particles are retained within the lymph vessels and nodes and do not readily leak out into nearby tissue and blood vessels. Thus, downstream lymph vessels, nodes and the thoracic duct maintain signal and continue to exhibit higher ICG fluorescence signal over time. At 72 h, ICG fluorescence was still observed in lymph nodes following ICG particle dosing while no lymph node fluorescence signal following free ICG dosing was detected (data not shown).

To quantify the differences in lymphatic exposure to ICG after SC dosing of free ICG or ICG particles, we measured the ICG fluorescence intensity at different locations along the mouse lymphatic system at each of the three post-injection time points (1, 5, 30 min). The structures in the lymphatics that were assayed, with their approximate distances away from the right hind foot SC injection site, are as follows: popliteal LN (2 cm), sciatic LN (2.5 cm), medial iliac LN (3.8 cm), lumbar LN (5.4 cm), inferior thoracic lymph duct (5.7 cm), medial thoracic lymph duct (6.5 cm) and superior thoracic lymph duct (7.3 cm). The ICG fluorescence intensities at each of these lymphatic structures at 1, 5 and 30 min following free ICG and ICG particle dosing are shown in Figure 3(C–E). For ICG particles, ICG fluorescence was consistently highest in all of these lymphatic structures at all three time points (Figure 3(C–E)). The drop in the fluorescence intensity in the sciatic LN (2.5 cm from the SC injection site) for both free ICG and ICG particles (Figure 3(C–E)) is due to the sciatic LN being deeper within muscle tissue and relatively small in volume compared to the other lymphatic structures analysed. These data confirm that ICG particles enable more rapid distribution of ICG throughout the lymphatic system and promote more extensive lymphatic retention of ICG and exposure to ICG than the soluble formulation of ICG.

Ability of ICG particles to verify small and abnormal lymph vessels

Leveraging the ability of ICG particles to be retained in lymph vessels with minimal penetration into nearby tissues (Figures 2 and 3), as well as undergo extensive lymphatic first-passage throughout the lymphatic network of vessels and nodes following SC

administration (Figure 3), we determined if the small lymph vessel connecting the subiliac and axillary LNs could be visualised. As schematically presented in Figures 1(C) and 4(A insert), ICG particles clearly delineated this lymph vessel adjacent to blood vessels, which were devoid of ICG signal (Figure 4(A)). This small mouse lymph vessel is estimated to be ~0.2 mm in diameter (Figure 4(A)). This lymph vessel was not visible in mice treated with control free ICG. In addition, the high degree of lymphatic resolution from ICG particles detected a lymph vessel abnormality in a 23-month old mouse that was resolved down to a level of approximately 5–10 cells in two dimensions *via* histopathology (Figure 4(B)). This abnormality was verified by histopathology to be an enlarged lymph vessel with significant perivascular mononuclear cell infiltrations (Figure 4(B insert)). Collectively, these data verify that ICG particles rapidly clear from the SC space, are preferentially taken up into the lymphatics from the SC space, and undergo widespread distribution and retention within the lymphatic system throughout the body prior to entering the blood at the lymph–blood anastomoses near the heart. These properties enabled delineation of small normal and lesioned lymph vessels.

Discussion

Unlike the free and soluble ICG counterpart, ICG particles exhibited rapid lymphatic uptake, retention and widespread distribution through the body within the lymphatic path between the foot SC injection site and the entry into the blood circulation by the heart (Figures 1–4(A)). These particles, likely due to the stable association of ICG to the particles, provided a high signal-to-noise ratio as a result of low surrounding tissue and blood background fluorescence signal, which enabled detection of a 0.2 mm thin and long lymph vessel connecting the subiliac and axillary LNs (Figure 4(A)). The high-resolution NIR imaging enabled by ICG particles also allowed detection of abnormal lymph vessels down to a resolution confirmed by histopathology to be approximately 5–10 cells (Figure 4(B)).

The detailed mechanisms of differential free/soluble versus nanoparticle ICG distribution from the SC space and the subsequent widespread lymphatic distribution and retention of ICG particles are not fully understood and remain to be investigated further. It is likely, however, that free/soluble small molecules (e.g. free ICG) in the SC space can penetrate across endothelial cells lining lymphatic or blood capillaries. Small molecules absorbed into the initial lymph capillaries in the SC space can egress back into the interstitium and be rapidly cleared into the systemic blood circulation by rapid blood flow in capillaries (~100–500-fold > than lymph flow in capillaries [27]). In contrast, the same molecules (i.e. ICG) stably bound to much larger nanoparticles are not permeable to blood capillaries but are permeable to lymph capillaries from the SC space. Once inside the lymph vessels, ICG particles remain in and distribute throughout the interconnected lymphatic system from local to distal LNs without leaking out of lymph vessels. As long as small molecules remain bound to nanoparticles and particles do not readily adsorb to lymph components, become endocytosed by cells in lymph vessels and nodes, or disintegrate, they circulate through lymph vessels and nodes before entering the blood at anastomoses between the thoracic lymphatic ducts and subclavian veins near the heart. This size-dependent differential blood versus lymphatic uptake–retention–distribution hypothesis is consistent with the observations that free ICG was taken up by lymphatics near the injection site (Figure 2(A–C,

G)), but did not travel extensively to downstream lymph vessels and nodes (Figure 3) and leaked out of lymphatics into proximal tissue and absorbed into the saphenous vein (blood circulation) (Figure 2(A–C, G)). The more extensive early entry into the blood circulation after free ICG dosing was confirmed by plasma ICG pharmacokinetics (Table 1), which to our knowledge is the first plasma PK data reported for ICG dosed SC in mice (IV has been the predominant administration route reported for ICG in mice [e.g. 10 mg/kg ICG dose] [28] and humans [e.g. 0.5–2.0 mg/kg ICG dose] [29]). Mice administered with ICG particles did not exhibit detectable plasma ICG in blood (Table 1); it is likely due to the stability of ICG particles that no free ICG was released from the lymph or the SC space and appeared in the blood, which would have been detectable in the plasma. This leakage of free ICG out of lymph vessels is consistent with studies that have shown healthy lymph vessels constitutively leak a portion of the fluid and solute (albumin) that they transport into the surrounding tissue [30]. In contrast, ICG particles did not diffuse to nearby tissues and blood vessels, but localised to and distributed widely throughout the lymphatics (Figures 2(D–F, H, I), 3 and 4(A)).

These data in mice are consistent with higher indinavir levels in multiple macaque lymph nodes (popliteal, inguinal, mesenteric, ileocecal, Peyer's patch, bronchial, axillary, tonsil and submandibular) after SC injection of the same lipid–drug nanoparticle formulation used in ICG particles compared to an oral free drug formulation [11]. Thus, it is clear that ICG particles enter and remain in lymph vessels, traversing from local to distal lymph nodes as their first-pass through the body without requiring blood recirculation to help promote widespread lymph node distribution. While additional cellular and molecular mechanisms remain to be elucidated and may contribute to lymph node accumulation of drug, the data collected in mice using free ICG and ICG particles are consistent with the above proposed mechanisms that drive the differential small-molecule solute and particle flow within the interconnected lymphatic vasculature.

Given the increased use of the SC route to reduce infection risk as well as healthcare cost of protein biotherapeutics intended for blood exposure [31], a fuller understanding of the role of molecular characteristics that drive distribution into the blood versus the lymph is needed. Molecular or particle size, shape, elasticity, surface properties (e.g. charge, hydrophilicity), receptor–ligand mediated processes at cell or tissue targets, opsonisation and even the injection site and injection volume, each individually or in combination, likely play a role in regulating blood versus lymphatic uptake, retention and distribution. Size, presented as hydrodynamic diameter of most globular proteins, has a significant impact on the differential diffusion rate across blood versus lymph vessels as well as through the SC space. For example, permeability ($p = 1$ – 0.01) through blood capillaries in human skeletal muscle has been reported to diminish with size (~ 0.3 – 5 nm): water (18 Da, ~ 0.3 nm) $p = 1.0$, glucose (180 Da, ~ 0.7 nm) $p = 0.6$, sucrose (342 Da, ~ 0.9 nm) $p = 0.4$, haemoglobin (64 kDa, ~ 5 nm) $p = 0.01$ [32]. Furthermore, leveraging the well-defined physicochemical characteristics of gadolinium (Gd) conjugated to dendrimer particles, effects of increasing particle size (G2-10, 4–14 nm) on blood vasculature (capillary) permeability were systematically investigated in mice using MRI. These studies found that the majority of Gd-dendrimers < 5 nm (G2-3) quickly leaked out of the vasculature into surrounding tissues, while most dendrimers > 8 nm (G6-10) showed minimal leakage from blood vessels [33]. These data are

consistent with the reported ~5–12 nm pore size of continuous and fenestrated (non-sinusoidal) human blood capillaries perfusing >95% of the body (e.g. skeletal muscle, skin) [34]. Recent mouse studies with larger 30nm magnetic particles given IV demonstrated almost exclusive blood vasculature retention in healthy mice but allowed extravascular tumour accumulation through leaky blood vessels [35].

These accumulated data support the hypothesis that molecules with hydrodynamic diameters >5–8 nm do not readily permeate in and out of blood vessels. In contrast, much larger molecules (even bacteria and viruses) can readily cross lymphatic capillaries from the SC space, partly due to the 2–3 μ m fenestrations between lymphatic endothelial cells [36]. As the estimated size of the interstitial water channels in the SC space is ~100 nm, molecules or particles should be <~100 nm to readily transit through these channels and gain access to lymphatic capillaries [27]. With ICG associated to particles ~60 nm in size, they are small enough to rapidly transit through the interstitial water channels, but are too large to penetrate blood capillaries; thus, they appeared predominantly in and were retained by lymph vessels. It is possible that the nanoparticles that penetrated into lymph vessels were carried subsequently by lymph flow and were retained by specific retentive forces within the lymphatic system. Elucidation of these processes, while important, is beyond the scope of this report and is a topic of future investigation.

In addition to molecular size-dependent differentiation of lymph and blood vessel penetration, there are other physiological differences between lymph and blood that could contribute to treatment effectiveness. It is well-documented that human blood capillaries exhibit a flow rate ~100–500-fold faster than lymph capillaries. Thoracic duct lymph flow has been reported to range from 0.001 to 0.013 ml/min in mice, 0.008 to 0.017 ml/min in rats and 0.67 to 2.5 ml/min in humans, and depends upon mobilisation (e.g. anaesthetised vs. ambulatory) [37]. Moreover, total blood volume is larger than lymph volume (~5 vs. ~3 L), and protein and lipid levels are higher in blood than lymph. Also total protein concentration is much higher in blood than in lymph fluid; lipoprotein and lipid levels in lymph are ~10% of those in plasma [38]. Since many orally administered HIV drugs such as the protease inhibitors indinavir, ritonavir and lopinavir are highly protein bound, a majority of the fraction of oral HIV drug molecules found in the blood are protein bound, which further retards their ability to penetrate and distribute into lymph capillaries and nodes *via* extravasation from the blood into the interstitium or *via* penetration through high endothelial venules (HEVs) in lymph nodes. As a result, the concentration of oral HIV drugs in lymph is expected to be lower than that in blood. This concept, referred to as lymphatic drug insufficiency (LDI), was initially proposed and validated in our 2003 report [7], and recently confirmed in prospective studies in HIV patients that showed LDI of oral small-molecule antiretrovirals [8–10]. This is consistent with other reports in preclinical models and humans that showed the anti-cancer small molecules methotrexate [39], doxorubicin [40] and platinum-based chemotherapeutics [41] displayed limited lymphatic availability after IV and SC injection. This can result in negative clinical outcomes, particularly for antiviral therapy, as LDI in HIV patients on oral drug therapy has been associated with persistent HIV-1 RNA in LNs [8].

Approaches to improve lymphatic drug exposure include a number of drug carrier platforms (e.g. nanoparticles, polymers and liposomes), or *in situ* association with endogenous lipoproteins, proteins or cells (leukocytes) with lymphotropic properties. Macromolecules and carriers can also be derivatized with targeting agents that bind to lymphatic targets. Recently, the US Food and Drug Administration approved [^{99m}Tc] Tilmanocept (*Lymphoseek*) for lymphatic mapping. Its polymeric dextran backbone that accumulates in draining LNs by selectively binding to mannose receptors (CD206) on macrophage and dendritic cell surfaces prevents lymphatic egress of soluble ^{99m}Tc into the SC space and subsequent blood uptake [42]. However, only local draining lymph nodes are visualised at low resolution with this agent. Other nanoparticles without targeting to macrophages also show very limited lymphatic distribution, primarily to local nodes and content redistributed to local blood vessels [18,19,21].

Even without selective binding, the design of ICG particles (based on the early discovery that 40–80nm black ink particles access the LN interstitium [43]) enables lymphatic homing from the SC space and widespread lymphatic retention/distribution (no blood redistribution is necessary) (Figure 3). Furthermore, no targeting moiety on the particle surface is required to enable high-resolution lymphatic imaging of LNs and vessels. The ability of ~60 nm ICG particles to distribute throughout and remain in lymph vessels (Figure 4(A)) when given SC is novel and could provide invaluable diagnostic and drug targeting (theranostic) possibilities. Full tissue distribution studies for ICG particles and free ICG are under our current investigation, and are beyond the scope of this report.

Using this approach that specifically accesses the lymph vasculature throughout the body, we can probe in detail the anatomy and physiological state of the lymphatics (e.g. extent of lymph vessel leakiness or diminished clearance due to tumours). Recently reported carbon nanoparticles intended for lymphatic mapping only collect in lymph nodes and do not provide detailed resolution of the interconnecting lymph vessels or the thoracic duct [22]. Other recent lymphatic targeting approaches using nanoparticles (~20–60 nm) relied on the blood (after IV administration) to distribute to multiple LNs in mice [44,45]. With high background and limited blood-to-lymph transfer of nanoparticles in these studies, the ability to resolve small lymph vessels was low and thus derivation of pharmacokinetic and physiologic parameters required to develop and validate *in silico* physiologically based pharmacokinetic (PBPK) models would be limited. Our novel lymphatic first-passage approach using ICG particles given SC is a simplified experimental process and would likely provide a higher signal-to-noise ratio, and thus more efficient derivation of key parameters. With some optimisation, the use of ICG particles could provide real-time, direct physiological measurements and could overcome the inability of other nanostructures to resolve small lymph vessels widely throughout the lymphatic network. This may enable physiological measurements without using more invasive procedures that can perturb the biologic system [40]. With higher resolution and more confident derivation of key parameters, validated PBPK models may serve to predict lymphatic drug disposition and accelerate therapeutic product development.

The *in vivo* stability of ICG particles appeared to be an essential characteristic. *In vivo* stability of ICG association to the nanoparticles is evident by widespread and specific

lymphatic distribution (Figure 3) and vascular retention (Figure 4(A)), suggesting these particles remained intact. Should small molecule contrast agents come apart in biological milieu, the dissociated molecules would behave as the solute free form and would leak out of the lymphatics, diminishing fluorescence signal and the signal-to-noise ratio, as shown in Figure 3. Stable binding between ICG and nanoparticles enabled detection of delicate (Figure 4(A)) and abnormal or inflamed lymph vessels (Figure 4(B)). To our knowledge, this is the first demonstration of ICG nanoparticles being used to detect lymphatic vessels with confirmed inflammatory cell accumulations.

Abnormal lymph vessels such as those detected can permit lymph leakage, which is associated with the development of obesity, atherosclerosis, oedema, type 2 diabetes, and is thought to facilitate cancer cell entry into these vessels [4,46–48]. Thus, the ICG molecular-binding stability of particles and preferential lymphatic uptake/retention and widespread lymphatic distribution characteristics may be useful for convenient, real-time early diagnosis of abnormal lymph vessels and occluded lymph flow. Lymph flow is central to how well the lymphatic system functions, since functional transport of fluid and cells through lymph vessels maintains a properly functioning immune system by facilitating interactions between antigen presenting cells (APCs and DCs) and cognate lymphocytes. Defective lymphatic transport has been linked to impaired reverse cholesterol transport from tissues [49] and exacerbates atherosclerosis [50,51]. In addition, ICG particles could be used to guide intranodal immunisation injections, which have been shown to be ~100-fold more potent for eliciting an immune response for peptide and DNA vaccines than the same vaccine administered SC [52]. Furthermore, since solid tumours commonly induce an expansion of the surrounding lymphatic network [47,53], and functional lymphatic vessels are restricted to tumour margins and peritumoural regions surrounding tumours [54], ICG particles could be used to detect the margins of tumours and tumour lymphatic networks. The limited depth range of noninvasive NIR diagnostic imaging with ICG particles may be enhanced by using a NIR-II imaging device that could detect ICG's fluorescence emission in the 1000–1600 nm range [55,56] (NIR-II wavelengths demonstrate reduced photon scattering and tissue autofluorescence compared to the traditional 700–900 nm NIR-I range [26]) or by complementing these particles with similar Gd particles and MRI [57] to provide high-resolution 3D imaging analysis for early diagnosis of lymphatic malignancies. Our novel Gd particles exhibit ~5–10-fold greater Gd particle accumulation in tumour-infiltrated LNs in mice and much higher resolution than any available Gd MRI contrast agent [58]. In addition, more sensitive imaging devices [59] could provide further enhancement of NIR imaging capabilities with these ICG particles. Also, with surface coating of targeting biomarkers on ICG or Gd particles, resolution of these particles for abnormal LNs and vessels could be enhanced further.

Conclusion

In summary, we have shown that the lymphatics can be accessed widely and selectively when small molecules are stably bound to novel nanoparticles introduced into the SC space. Unlike parent soluble small molecules that readily permeated blood and lymph vessels, the same molecules bound to and stably associated with particles were preferentially taken up and retained by lymph vessels and nodes. These robust tracer nanoparticles allow high-

resolution (~0.2 mm, 5–10 cells) NIR imaging of superficial lymph vessels and nodes and show higher resolving power of lymphatic anatomy than free ICG. Collectively, the properties of these particles may be used to diagnose as well as treat lymphatic abnormalities – particularly cancer metastases and residual HIV – by enhancing drug exposure in lymph nodes and vessels. These particles may also be further developed to specifically interact with cell populations and signalling pathways in the lymphatic system to mediate immune responses.

Acknowledgments

We thank Brian Johnson, Erin McCarty and the UW Histology and Imaging Core (HIC) for histology support and the members of the NIH-funded Targeted Long-Acting Combination Anti-Retroviral Therapy (TLC-ART) Program who contributed insights through many helpful discussions.

Funding

This work was supported by the National Institutes of Health [grant numbers UM1 AI120176, T32-GM007750, TL1-1TR002318-01 and 1S10OD010652-01].

References

1. Levick JR, Michel CC. Microvascular fluid exchange and the revised Starling principle. *Cardiovasc Res.* 2010; 87:198–210. [PubMed: 20200043]
2. Trevaskis NL, Kaminskas LM, Porter CJ. From sewer to saviour – targeting the lymphatic system to promote drug exposure and activity. *Nat Rev Drug Discov.* 2015; 14:781. [PubMed: 26471369]
3. Ruddle NH. Lymphatic vessels and tertiary lymphoid organs. *J Clin Invest.* 2014; 124:953–959. [PubMed: 24590281]
4. Alitalo K. The lymphatic vasculature in disease. *Nat Med.* 2011; 17:1371–1380. [PubMed: 22064427]
5. Estes JD. Pathobiology of HIV/SIV-associated changes in secondary lymphoid tissues. *Immunol Rev.* 2013; 254:65–77. [PubMed: 23772615]
6. Chen J, Wang L, Yao Q, et al. Drug concentrations in axillary lymph nodes after lymphatic chemotherapy on patients with breast cancer. *Breast Cancer Res.* 2004; 6:R474–R477. [PubMed: 15217515]
7. Kinman L, Brodie SJ, Tsai CC, et al. Lipid-drug association enhanced HIV-1 protease inhibitor indinavir localization in lymphoid tissues and viral load reduction: a proof of concept study in HIV-2287-infected macaques. *J Acquir Immune Defic Syndr.* 2003; 34:387–397. [PubMed: 14615656]
8. Fletcher CV, Staskus K, Wietgreffe SW, et al. Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. *Proc Natl Acad Sci USA.* 2014; 111:2307–2312. [PubMed: 24469825]
9. Lee S, Hatano H, Kashuba AD, et al. Conference on retroviruses and opportunistic infections (CROI). Seattle, WA: 2017. Integrase and protease inhibitor concentrations in lymph node and gut mucosal tissue (Abstract #407). 2017
10. Lorenzo-Redondo R, Fryer HR, Bedford T, et al. Persistent HIV-1 replication maintains the tissue reservoir during therapy. *Nature.* 2016; 530:51–56. [PubMed: 26814962]
11. Freeling JP, Ho RJ. Anti-HIV drug particles may overcome lymphatic drug insufficiency and associated HIV persistence. *Proc Natl Acad Sci USA.* 2014; 111:E2512–E2513. [PubMed: 24889644]
12. Freeling JP, Koehn J, Shu C, et al. Long-acting three-drug combination anti-HIV nanoparticles enhance drug exposure in primate plasma and cells within lymph nodes and blood. *Aids.* 2014; 28:2625–2627. [PubMed: 25102089]

13. Kraft JC, McConnachie LA, Koehn J, et al. Long-acting combination anti-HIV drug suspension enhances and sustains higher drug levels in lymph node cells than in blood cells and plasma. *Aids*. 2017; 31:765–770. [PubMed: 28099191]
14. Kraft JC, Ho RJ. Interactions of indocyanine green and lipid in enhancing near-infrared fluorescence properties: the basis for near-infrared imaging in vivo. *Biochemistry*. 2014; 53:1275–1283. [PubMed: 24512123]
15. Frangioni JV. In vivo near-infrared fluorescence imaging. *Curr Opin Chem Biol*. 2003; 7:626–634. [PubMed: 14580568]
16. Nguyen QT, Tsien RY. Fluorescence-guided surgery with live molecular navigation – a new cutting edge. *Nat Rev Cancer*. 2013; 13:653–662. [PubMed: 23924645]
17. Kinman L, Bui T, Larsen K, et al. Optimization of lipid-indinavir complexes for localization in lymphoid tissues of HIV-infected macaques. *J Acquir Immune Defic Syndr*. 2006; 42:155–161. [PubMed: 16760797]
18. Davies-Venn CA, Angermiller B, Wilganowski N, et al. Albumin-binding domain conjugate for near-infrared fluorescence lymphatic imaging. *Mol Imaging Biol*. 2012; 14:301–314. [PubMed: 21688052]
19. Proulx ST, Luciani P, Christiansen A, et al. Use of a PEG-conjugated bright near-infrared dye for functional imaging of rerouting of tumor lymphatic drainage after sentinel lymph node metastasis. *Biomaterials*. 2013; 34:5128–5137. [PubMed: 23566803]
20. Polom K, Murawa D, Rho YS, et al. Current trends and emerging future of indocyanine green usage in surgery and oncology: a literature review. *Cancer*. 2011; 117:4812–4822. [PubMed: 21484779]
21. Burrows PE, Gonzalez-Garay ML, Rasmussen JC, et al. Lymphatic abnormalities are associated with RASA1 gene mutations in mouse and man. *Proc Natl Acad Sci USA*. 2013; 110:8621–8626. [PubMed: 23650393]
22. Xie P, Xin Q, Yang ST, et al. Skeleton labeled ¹³C-carbon nanoparticles for the imaging and quantification in tumor drainage lymph nodes. *Int J Nanomedicine*. 2017; 12:4891–4899. [PubMed: 28744123]
23. Zhang F, Niu G, Lu G, et al. Preclinical lymphatic imaging. *Mol Imaging Biol*. 2011; 13:599–612. [PubMed: 20862613]
24. Smedley J, Turkbey B, Bernardo ML, et al. Tracking the luminal exposure and lymphatic drainage pathways of intravaginal and intrarectal inocula used in nonhuman primate models of HIV transmission. *PLoS One*. 2014; 9:e92830. [PubMed: 24667371]
25. Verbeek FP, Troyan SL, Mieog JS, et al. Near-infrared fluorescence sentinel lymph node mapping in breast cancer: a multicenter experience. *Breast Cancer Res Treat*. 2014; 143:333–342. [PubMed: 24337507]
26. Hong G, Antaris AL, Dai H. Near-infrared fluorophores for biomedical imaging. *Nat Biomed Eng*. 2017; 1doi: 10.1038/s41551-016-0010
27. Wiig H, Swartz MA. Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. *Physiol Rev*. 2012; 92:1005–1060. [PubMed: 22811424]
28. Milesi-Halle A, Abdel-Rahman SM, Brown A, et al. Indocyanine green clearance varies as a function of N-acetylcysteine treatment in a murine model of acetaminophen toxicity. *Chem Biol Interact*. 2011; 189:222–229. [PubMed: 21145883]
29. Meijer DK, Weert B, Vermeer GA. Pharmacokinetics of biliary excretion in man. VI. Indocyanine green. *Eur J Clin Pharmacol*. 1988; 35:295–303. [PubMed: 3181282]
30. Scallan JP, Huxley VH. In vivo determination of collecting lymphatic vessel permeability to albumin: a role for lymphatics in exchange. *J Physiol*. 2010; 588:243–254. [PubMed: 19917564]
31. Pivot X, Gligorov J, Muller V, et al. Preference for subcutaneous or intravenous administration of trastuzumab in patients with HER2-positive early breast cancer (PrefHer): an open-label randomised study. *Lancet Oncol*. 2013; 14:962–970. [PubMed: 23965225]
32. Pappenheimer JR. Passage of molecules through capillary walls. *Physiol Rev*. 1953; 33:387–423. [PubMed: 13088295]
33. Kobayashi H, Brechbiel MW. Dendrimer-based macromolecular MRI contrast agents: characteristics and application. *Mol Imaging*. 2003; 2:1–10. [PubMed: 12926232]

34. Sarin H. Physiologic upper limits of pore size of different blood capillary types and another perspective on the dual pore theory of microvascular permeability. *J Angiogenesis Res.* 2010; 2:14. [PubMed: 20701757]
35. Miller MA, Gadde S, Pfirschke C, et al. Predicting therapeutic nanomedicine efficacy using a companion magnetic resonance imaging nanoparticle. *Sci Transl Med.* 2015; 7:314ra183.
36. Baluk P, Fuxe J, Hashizume H, et al. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med.* 2007; 204:2349–2362. [PubMed: 17846148]
37. Lindena J, Kupper W, Trautschold I. Catalytic enzyme activity concentration in thoracic duct, liver, and intestinal lymph of the dog, the rabbit, the rat and the mouse. Approach to a quantitative diagnostic enzymology, II. Communication. *J Clin Chem Clin Biochem.* 1986; 24:19–33. [PubMed: 3701268]
38. Nanjee MN, Cooke CJ, Olszewski WL, et al. Lipid and apolipoprotein concentrations in prenodal leg lymph of fasted humans. Associations with plasma concentrations in normal subjects, lipoprotein lipase deficiency, and LCAT deficiency. *J Lipid Res.* 2000; 41:1317. [PubMed: 10946020]
39. Kaminskas LM, McLeod VM, Ascher DB, et al. Methotrexate-conjugated PEGylated dendrimers show differential patterns of deposition and activity in tumor-burdened lymph nodes after intravenous and subcutaneous administration in rats. *Mol Pharm.* 2015; 12:432–443. [PubMed: 25485615]
40. Ryan GM, Kaminskas LM, Bulitta JB, et al. PEGylated polylysine dendrimers increase lymphatic exposure to doxorubicin when compared to PEGylated liposomal and solution formulations of doxorubicin. *J Control Release.* 2013; 172:128–136. [PubMed: 23954628]
41. Zor M, Yildirim I, Basal S, et al. Intralymphatic delivery of platinum-based chemotherapeutics is possible: an experimental study. *J Cancer Res Clin Oncol.* 2012; 138:1679–1682. [PubMed: 22638885]
42. Wallace AM, Han LK, Povoski SP, et al. Comparative evaluation of [(99m)tc]tilmanocept for sentinel lymph node mapping in breast cancer patients: results of two phase 3 trials. *Ann Surg Oncol.* 2013; 20:2590–2599. [PubMed: 23504141]
43. Drinker CK, Field ME, Ward HK. The filtering capacity of lymph nodes. *J Exp Med.* 1934; 59:393–405. [PubMed: 19870253]
44. Pu K, Shuhendler AJ, Jokerst JV, et al. Semiconducting polymer nanoparticles as photoacoustic molecular imaging probes in living mice. *Nat Nanotechnol.* 2014; 9:233–239. [PubMed: 24463363]
45. Xiong L, Shuhendler AJ, Rao J. Self-luminescing BRET-FRET near-infrared dots for in vivo lymph-node mapping and tumour imaging. *Nat Commun.* 2012; 3:1193. [PubMed: 23149738]
46. Scallan JP, Hill MA, Davis MJ. Lymphatic vascular integrity is disrupted in type 2 diabetes due to impaired nitric oxide signalling. *Cardiovasc Res.* 2015; 107:89–97. [PubMed: 25852084]
47. Stacker SA, Williams SP, Karnezis T, et al. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nat Rev Cancer.* 2014; 14:159–172. [PubMed: 24561443]
48. Harvey NL, Srinivasan RS, Dillard ME, et al. Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. *Nat Genet.* 2005; 37:1072–1081. [PubMed: 16170315]
49. Lim HY, Thiam CH, Yeo KP, et al. Lymphatic vessels are essential for the removal of cholesterol from peripheral tissues by SR-BI-mediated transport of HDL. *Cell Metab.* 2013; 17:671–684. [PubMed: 23663736]
50. Martel C, Li W, Fulp B, et al. Lymphatic vasculature mediates macrophage reverse cholesterol transport in mice. *J Clin Invest.* 2013; 123:1571–1579. [PubMed: 23524964]
51. Randolph GJ, Miller NE. Lymphatic transport of high-density lipoproteins and chylomicrons. *J Clin Invest.* 2014; 124:929–935. [PubMed: 24590278]
52. Maloy KJ, Erdmann I, Basch V, et al. Intralymphatic immunization enhances DNA vaccination. *Proc Natl Acad Sci USA.* 2001; 98:3299–3303. [PubMed: 11248073]
53. Skobe M, Hawighorst T, Jackson DG, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med.* 2001; 7:192–198. [PubMed: 11175850]

54. Padera TP, Kadambi A, di Tomaso E, et al. Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science*. 2002; 296:1883–1886. [PubMed: 11976409]
55. Starosolski Z, Bhavane R, Ghaghada KB, et al. Indocyanine green fluorescence in second near-infrared (NIR-II) window. *PLoS One*. 2017; 12:e0187563. [PubMed: 29121078]
56. Carr JA, Franke D, Caram JR, et al. Shortwave infrared fluorescence imaging with the clinically approved near-infrared dye indocyanine green. *bioRxiv*. 2017; doi: 10.1101/100768
57. Bui T, Stevenson J, Hoekman J, et al. Novel Gd nanoparticles enhance vascular contrast for high-resolution magnetic resonance imaging. *PLoS One*. 2010; 5:e13082. [PubMed: 20927340]
58. Partridge SC, Kurland BF, Liu CL, et al. Tumor-induced lymph node alterations detected by MRI lymphography using gadolinium nanoparticles. *Sci Rep*. 2015; 5:15641. [PubMed: 26497382]
59. Zhu B, Sevik-Muraca EM. A review of performance of near-infrared fluorescence imaging devices used in clinical studies. *Br J Radiol*. 2015; 88:20140547. [PubMed: 25410320]

Figure 1.

Comparison of free ICG, ICG particle and quantum dot (QD) clearance from the SC foot injection site. (A) Representative NIR fluorescence images of the injection site ($N = 3-5$). (B) Time course of the foot SC injection site NIR fluorescence intensity (arbitrary units, a.u.). Rate constants (min⁻¹) and R^2 values for trendlines are listed. (C) Schematic representation of the superficial lymphatic vessels and nodes (popliteal, sciatic, subiliac and axillary) on the right side of the mouse in relation to the dorsal right hind foot SC injection site.

Figure 2.

Comparison of ICG localisation following SC dosing of free ICG or ICG particles. NIR fluorescence images (top three rows) and corresponding bright field images with false colour fluorescence overlay (bottom row) of the *ventral* right hind leg muscle tissue and saphenous vein (arrows) with skin removed 1, 5, 30 and 60 min post-SC foot injection of (A–C, G) free ICG or (D–F, H, I) ICG particles.

Figure 3.

Time course of ICG distribution in the lymphatic system following a single SC dose of free ICG or ICG particles. NIR fluorescence images (top) and corresponding bright field images with false colour fluorescence overlay (bottom) showing the lymphatic distribution of ICG at 1, 5 and 30 min following SC dosing of (A) free ICG or (B) ICG particles. Comparison of the ICG fluorescence intensity versus distance from the SC injection site of the lymph node or thoracic lymph duct at (C) 1 min, (D) 5 min and (E) 30 min following SC injection of free ICG (open circles) or ICG particles (solid circles). The structures in the lymphatics that were assayed and their approximate distance away from the right hind foot SC injection site, which is listed on the x-axis of (C–E), are as follows: popliteal LN (2.0 cm), sciatic LN (2.5 cm), medial iliac LN (3.8 cm), lumbar LN (5.4 cm), inferior thoracic lymph duct (5.7 cm), medial thoracic lymph duct (6.5 cm) and superior thoracic lymph duct (7.3 cm). *, p values < .05.

Figure 4.

(A) ICG particles detected a small lymph vessel adjacent to blood vessels. NIR fluorescence image of mouse skin opened with ICG particles post-SC foot injection tracing a small lymph vessel (stemmed arrows) connecting the right subiliac and axillary LNs without egressing from the lymph vessel into nearby tissue or the parallel dark blood vessel (non-stemmed arrows). Scale bar, 3 mm. (B) Lymphatic retention of ICG particles permitted detection of a lymphatic abnormality. NIR fluorescence image of ICG particles in the hind limbs of a mouse exhibiting abnormal, enlarged afferent popliteal lymph vessels (*). H&E histology (top) and anti-podoplanin immunohistochemistry (bottom) of control (left) and abnormal (right) lymph vessels that correspond to the locations in the mouse indicated by arrows. Histological analysis shows perivascular and intravascular mononuclear cell accumulations for the abnormal lymph vessels (right) but not for the normal lymph vessels (left).

Table 1

Early time course of indocyanine green (ICG) concentration (ng/mL) in mouse plasma following a single subcutaneous dose of ICG (3.0 mg/kg) formulated as either free ICG or ICG particles.

Time (min)	ICG plasma conc. (ng/mL)	
	Free ICG	ICG particles
2.5	61.03 ± 8.50	BLOQ
5	74.06 ± 29.93	BLOQ

Arithmetic mean ± SD of 3–4 mice per time point. BLOQ, below limit of quantification. Free ICG and ICG particles were dosed at 3.0 mg/kg ICG *via* identical SC injection volumes to the inside of the left hind leg.