Host–guest interactions of indocyanine green with β-cyclodextrin permit real-time characterization of the rat lymphatic system

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ABSTRACT

Objective: Fluorescence contrast technology using indocyanine green (ICG) could be useful for the rapid, dynamic, and objective assessment of blood vessels and the surrounding tissues when combined with near-infrared (NIR) imaging. Although ICG is a clinically available NIR fluorescence imaging probe, it can easily aggregate and is, thus, unstable. In the present study, we examined the efficacy of a host–guest ICG–β-cyclodextrin (CD) complex, which is used in pharmaceutics to improve the water solubility, stability, and bioavailability of hydrophobic molecules, for NIR imaging after hind footpad administration in a rat model.

Methods: To verify the performance of the ICG-β-CD complex with the host–guest self-assembly method in vivo, we performed simultaneous small animal (IVIS Spectrum system; PerkinElmer, Waltham, MA) and clinical (DIGI-MIH-001 near-infrared fluorescence imaging system; Beijing Digital Precision Medical Technology Co, Ltd, Beijing, China) imaging and evaluated the fluorescent properties of the ICG-β-CD complex in the hind footpad model of Sprague-Dawley male rats.

Results: We successfully prepared the ICG-β-CD complex. Compared with ICG, in vivo experiments showed that this complex had reduced absorbance at 710 nm and increased absorbance at 780 nm, indicating that it could prevent the dimeric aggregation of ICG, and a significantly higher fluorescence intensity at 730 nm excitation. After injection of 1.25 mg/mL of ICG or ICG-β-CD complex solutions into the rat hind footpad, fluorescent NIR lymphatic images were observed with both imaging systems. During the 12-hour observation period, the signal background ratio of ICG-β-CD showed a greater acute increase and a higher signal background ratio compared with ICG. The signal background ratio of ICG-β-CD was 125 to 100 from 260 to 540 minutes. These in vivo data suggest that ICG-β-CD has greater diffusion from the injection site and faster transport to the lymphatic system compared with ICG.

Conclusions: ICG-β-CD showed faster lymphatic transport than ICG, allowing for more rapid lymphatic NIR imaging. Thus, the ICG-β-CD complex might be a promising fluorescent agent for clinical lymphatic NIR imaging. (JVS–Vascular Science 2022;3:211-8.)

Clinical Relevance: The lymphatic system plays a crucial role in maintaining tissue fluid homeostasis by draining protein-rich fluid from the perivascular interstitial spaces back into the circulation. The lymphatic system also plays a variety of roles in the progression of some peripheral vascular diseases, including venous leg ulcers, atherosclerotic vascular disease, and severe foot infection. Understanding the dynamic changes of the lymphatic fluid is indispensable for a variety of clinical situations and research areas. We investigated the potential feasibility of the indocyanine green–β-cyclodextrin complex in clinical applications using clinically available near-infrared fluorescence imaging equipment.

Keywords: β-Cyclodextrin; Indocyanine green; Lymphatic circulation; Near-infrared fluorescence imaging

The lymphatic system plays a crucial role in maintaining tissue fluid homeostasis by draining protein-rich fluid from the perivascular interstitial spaces back into the circulation.[1] Additionally, the lymphatic system is responsible for absorbing lipids from the intestine and transporting leukocytes and antigen-presenting cells from

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inflammatory tissues.[2] Recent studies have also demonstrated a variety of roles for the lymphatic system in the progression of various peripheral vascular diseases, including venous leg ulcers, atherosclerotic vascular disease, and severe foot infection.[3–5] Thus, understanding the dynamic changes in lymphatic fluid is important for a range of clinical situations and research fields.

Traditional clinical in vivo lymphatic imaging techniques, including lymphangiography, magnetic resonance imaging, computed tomography lymphangiography, and lymphoscintigraphy, have a range of disadvantages, including invasiveness, complex operation, radiation exposure, and a lack of real-time dynamic imaging. Indocyanine green (ICG) fluorescence lymphatic imaging is a promising radiation-free, nonmicroscopic molecular imaging technology, which can provide real-time lymphatic images inside tissues, and is the most widely used imaging technique in the peripheral vascular field.[6–8] ICG is a relatively nontoxic fluorescent iodide dye that has been used in medical applications since the 1950s.[9] However, because of its sulfonyl group, the fluorescent iodide dye of ICG is unstable and forms dimers and oligomers in aqueous solutions. These aggregates result in self-quenching of the ICG, reducing its fluorescence in water.[10,11] The incorporation of ICG into various nanomaterials has been widely used to improve ICG stability, promote its drainage from perivascular interstitial spaces, and, thus, improve its target site accumulation.[12,13] Nevertheless, because the biocompatibility of a molecule is critical for its clinical utility, an alternative method to improve ICG biocompatibility involves protecting it from an aqueous environment via inclusion complexation with nontoxic β-cyclodextrin (CD) molecules.

In the present study, we examined the utility of a host–guest fluorescent contrast agent for lymphatic imaging in the rat, which was determined by the host–guest interaction between ICG and β-CD (Fig 1).[14] We also investigated the potential feasibility of the ICG-β-CD complex in clinical applications using near-infrared (NIR) fluorescence imaging.

ARTICLE HIGHLIGHTS

- **Type of Research:** Investigation of the efficacy of a host–guest indocyanine green (ICG)–β-cyclodextrin (CD) complex in a rat model
- **Key Findings:** We successfully prepared the ICG-β-CD complex, which showed faster lymphatic transport than ICG alone, allowing for more rapid lymphatic near-infrared imaging. Thus, the ICG-β-CD complex might be a promising fluorescent agent for clinical lymphatic near-infrared imaging.
- **Take Home Message:** We performed simultaneous small animal (IVIS Spectrum System; PerkinElmer, Waltham, MA) and clinical (DIGI-MIH-001 near-infrared fluorescence imaging system; Beijing Digital Precision Medical Technology Co, Ltd, Beijing, China) imaging studies and evaluated the fluorescent properties of the ICG-β-CD complex in a hind footpad model of Sprague-Dawley male rats.

METHODS

Materials. ICG was obtained from Dandong Yichuang Pharmaceutical Co, Ltd (Dandong, China). β-CD was purchased from the Sigma-Aldrich Co, Ltd (St Louis, MO). Methanol was obtained from the Beijing Chemical Reagent Co (Beijing, China). All solvents and chemicals were used without further purification.

Synthesis of the ICG-β-CD complex. The ICG-β-CD complex was prepared using a host–guest self-assembly process.[14,15] ICG (1.0 mg) and β-CD (3.0 mg) were completely dissolved in 0.5 mL of methanol and 1.5 mL of deionized water, respectively. The ICG solution was then added to the β-CD aqueous solution in drops to obtain the ICG-β-CD complex, followed by stirring for >6 hours. The mixture was further dialyzed against deionized water using a 1-kDa molecular weight cutoff membrane for 3 days to remove free ICG, β-CD monomers, and residual methanol.

Optical properties of ICG and the ICG-β-CD complex. The ultraviolet-visible spectroscopy absorption spectra of ICG and the ICG-β-CD complex were analyzed using spectrophotometry (UV-2700; Shimadzu Co, Kyoto, Japan) at 298 K. The wavelength range was from 400 to 900 nm. The fluorescence emission spectra of free ICG and the ICG-β-CD complex were analyzed using a microplate reader (Bio-Tek Instruments, Winooski, VT) at an excitation wavelength of 730 nm and emission wavelengths ranging from 750 to 900 nm at 298 K.

NIR fluorescence imaging in vivo. Real-time animal imaging was performed using two fluorescence imaging systems—the IVIS Spectrum small animal three-dimensional real-time imaging system (PerkinElmer, Waltham, MA) and the DIGI-MIH-001 clinical NIR fluorescence imaging system (Beijing Digital Precision Medical Technology, Co, Ltd, Beijing, China).

Experimental animal model and surgical procedures. Our local animal ethics committee approved the animal study protocol, which followed the regulations for animal experiments of the Chinese PLA General Hospital. A total of 24 Sprague-Dawley male rats (age, 12 weeks; mean weight, 390 g) were housed in a temperature- and humidity-controlled environment with free access to food and water until the experiments. All surgical procedures and imaging studies were performed with continuous isoflurane inhalation anesthesia using a small animal anesthesia machine. During the anesthesia

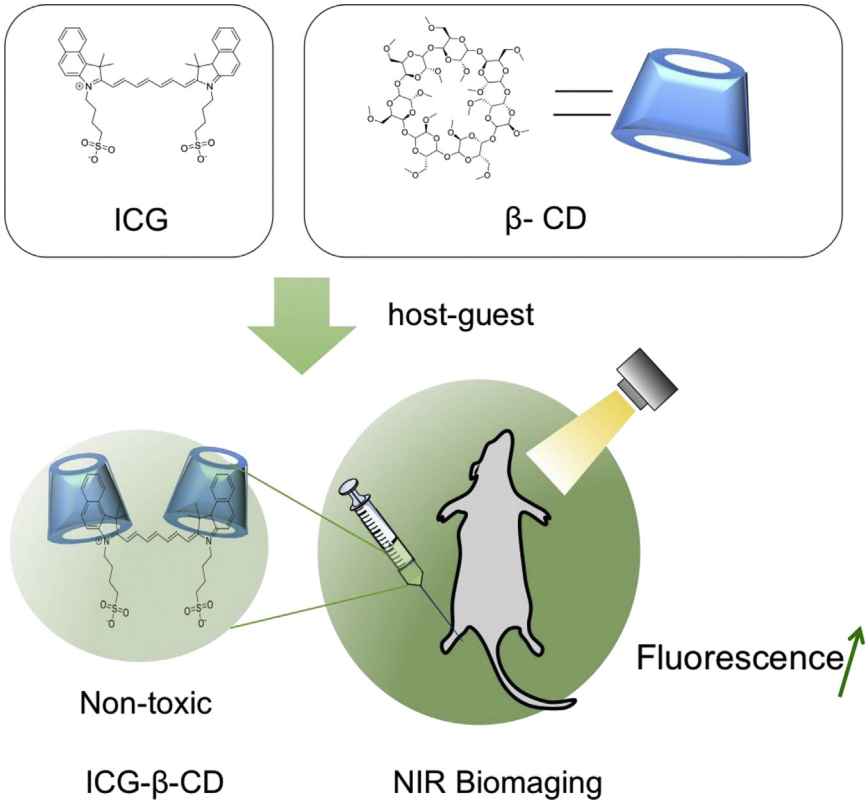

Fig 1. Scheme of host—guest formation between indocyanine green (ICG) and ICG-β-cyclodextrin (ICG-β-CD) in an aqueous state for enhanced fluorescence-guided imaging. *NIR*, near-infrared. Redrawn from Jo et al.[14]

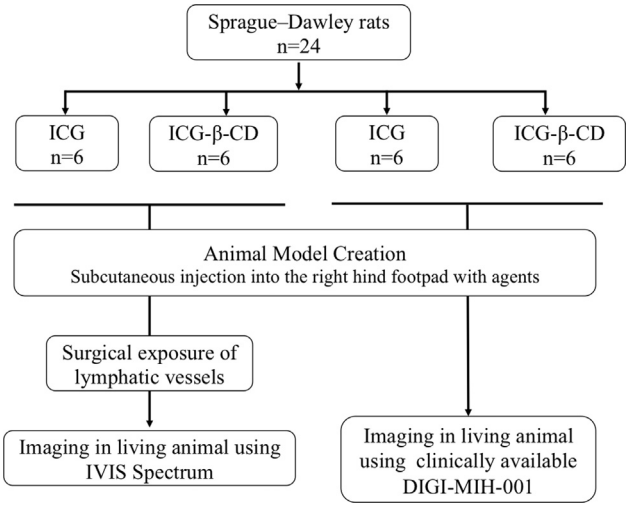

Fig 2. The study flowchart. *ICG,* Indocyanine green; *ICG-β-CD,* indocyanine green—β-cyclodextrin.

periods, the rats were placed on a heating pad maintained at 32°C and were administered lactated Ringer's solution (10 mL/kg, intraperitoneal injection) at 1- to 2-hour intervals.

The in vivo experiments in the present study consisted of two parts (Fig 2). The first part involved imaging using the IVIS spectrum system. With the rat in the supine position, the femoral vessel and accompanying lymphatics were exposed via a longitudinal incision from the medial thigh to the groin.[12] ICG or ICG-β-CD solution (1.25 mg/mL; 0.2 mL) were subcutaneously injected into the hind footpad using a 32-gauge needle. After injection, the rats were observed every 5 minutes for 60 minutes. The second part involved imaging using the clinical DIGI-MIH-001 system. The hair was removed from the lower abdomen and hindlimb area of the rats, and the imaging was performed without a skin incision. The rats were injected as described. To assess the clearance of ICG and the ICG-β-CD complex from the injection site, the right ankle was selected as the region of interest to measure the signal background ratio (SBR). NIR imaging was performed every 5 minutes for the first 60 minutes, every 15 minutes for the next 3 hours, and every 30 minutes for the final 8 hours.

All data are presented as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism, version 6 (GraphPad Software, Inc, La Jolla, CA). A P value $< .05$ was considered statistically significant. For two groups with repeated observations over time, two-way repeated measures analysis of variance with Sidak's multiple comparisons test was initially performed. A potential nonlinear association between the

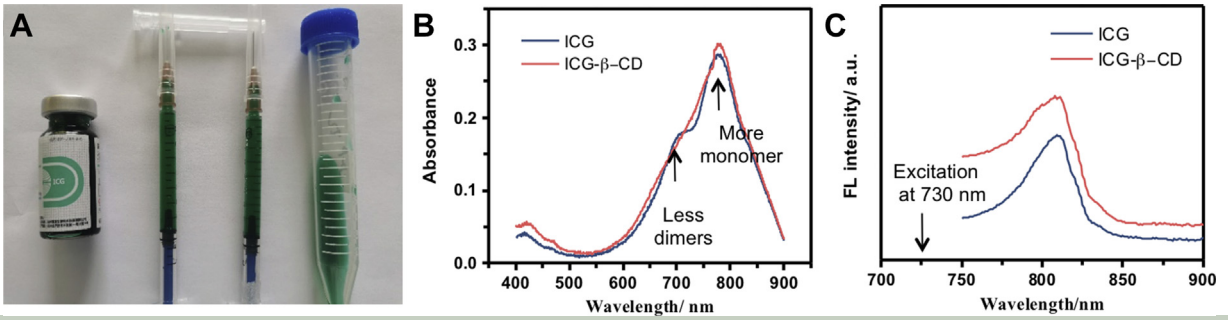

Fig 3. Optical properties of indocyanine green (ICG) and ICG-β-cyclodextrin (ICG-β-CD). **A,** Optical images of ICG and ICG-β-CD at 1.25 mg/mL. **B,** Absorbance spectra of ICG and ICG-β-CD. **C,** Fluorescence spectra of ICG and ICG-β-CD solutions on excitation at 730 nm.

time and SBR was explored using curve fitting with a third-order polynomial equation (cubic) and evaluated using the extra-sum-of-squares F test. Mixed models with the interaction of time and a grouping factor were used to compare the change in SBR over time between the two groups, which were implemented in the statistical software packages R (R Foundation for Statistical Computing, Vienna, Austria; available at: http://www.R-project.org) and EmpowerStats (X&Y Solution, Inc, Boston, MA; available at: http://www.empowerstats.com).

RESULTS

Optimal conditions for ICG and ICG-β-CD complex formation. The 1.25 mg/mL ICG and ICG-β-CD solutions are shown in Fig 3, *A*. The absorption peak of the ICG-β-CD complex was largely similar to that of ICG between the wavelengths of 400 and 900 nm (Fig 3, *B*). The absorbance of the ICG-β-CD complex at 710 nm was reduced and at 780 nm was increased compared with free ICG, indicating that ICG-β-CD reduced the dimeric aggregation of ICG. The ICG-β-CD complex also showed a greater fluorescence intensity than ICG after excitation at 730 nm (Fig 3, *C*). The results of the ICG-β-CD stability experiments showed that the fluorescence intensity remained unchanged for 14 days. The absorbance spectra of ICG-β-CD at 730 nm was ~0.15 (Supplementary Fig). These data indicated that the prepared ICG-β-CD complex has high optical and chemical stability.

Lymphatic transport efficacy of the ICG-β-CD complex using clinically available NIR fluorescence imaging. Both ICG and ICG-β-CD can be used to visualize the lymphatic system. Using the host—guest reaction, ICG-β-CD improved the stability of the fluorescent group of the ICG molecule and enhanced its imaging performance in the lymphatic system. The fluorescence intensity of lymph node imaging increased within 2 hours of ICG-β-CD injection (Fig 4). Histologic observation using hematoxylin and eosin staining confirmed the fluorescence imaging findings.

Assessment of acute in vivo fluorescence using the IVIS spectrum system. The imaging of ICG and the ICG-β-CD complex was performed from 5 minutes to 1 hour after hind footpad administration (Fig 5). The ICG-β-CD complex showed a trend toward faster drainage via the lymphatic system, indicating greater diffusion of the ICG-β-CD complex to the liver and circulation through the lymphatic system. The ICG-β-CD complex was rapidly transported via the lymphatics to the liver, with the initial fluorescent signal appearing after 15 minutes.

Assessment of diffusion properties of the ICG-β-CD complex using the clinical DIGI-MIH-001 system. The ICG-β-CD complex demonstrated different in vivo features compared with ICG, with faster lymphatic transport and faster removal at the subcutaneous injection site. The NIR images of the deep lymphatic vessels were visualized using DIGI-MIH-001 at 1 hour after injection of ICG or the ICG-β-CD complex into the right hind footpad (Fig 6).

The relationships of SBR over time for the ICG and ICG-β-CD groups were nonlinear (nonlinear vs linear model, $P < .01$ for both groups; Fig 7). Two-way repeated measures analysis of variance showed significant differences between the two groups (interaction: time × grouping factor, $P < .01$). The SBR in the ICG-β-CD group increased rapidly during the initial 4 hours and then remained relatively flat. In contrast, the SBR in the ICG group had increased slowly for the first 8 hours. The change in SBR was significantly greater in the ICG-β-CD group compared with the ICG group from 0 to 6 hours (slope difference, 25.65; standard error, 11.93; $P = .03$) and at 4 hours (slope difference, 59.95; standard error, 11.93; $P < .01$) after the footpad injection. These data suggest that the ICG-β-CD complex had faster diffusion properties than ICG after footpad injection.

DISCUSSION

A key finding of the present study was that ICG-β-CD had faster lymphatic transport than did ICG, allowing for faster lymphatic NIR imaging. Additionally, the

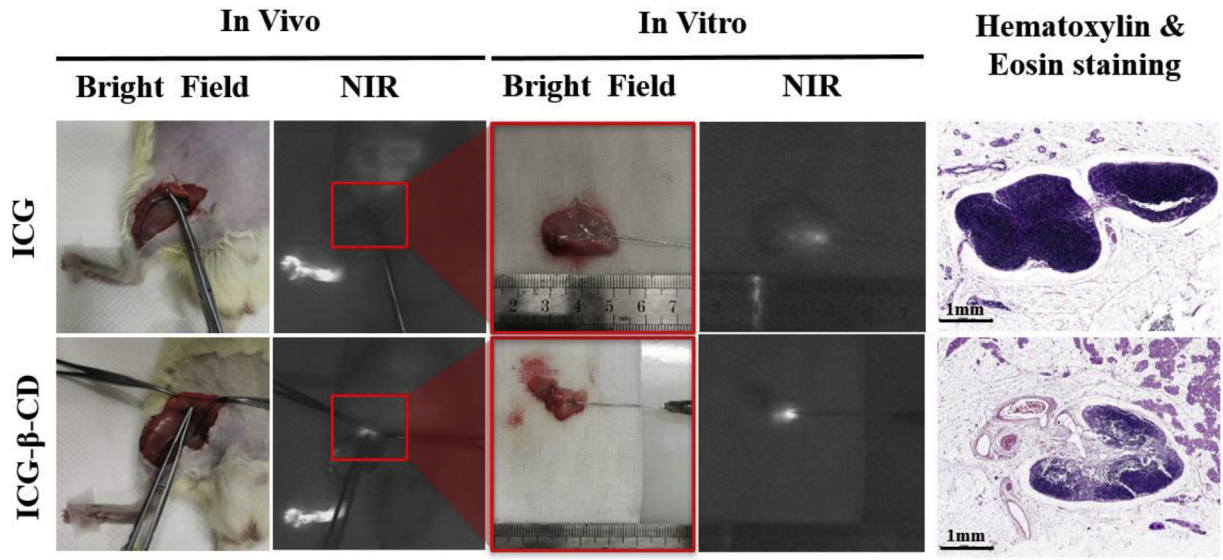

Fig 4. In vivo real-time fluorescence imaging using DIGI-MIH-001 and histologic staining confirmed enrichment of both fluorescent dyes at the inguinal lymph node location at 2 hours after injection in rats. The *red box* in the second panel from the left shows the in situ soft tissue with high brightness in near-infrared (NIR) imaging. The soft tissues in the third and fourth panels show the fatty tissue with the lymph node dissected out. The fifth panel shows the hematoxylin and eosin staining of the lymph node.

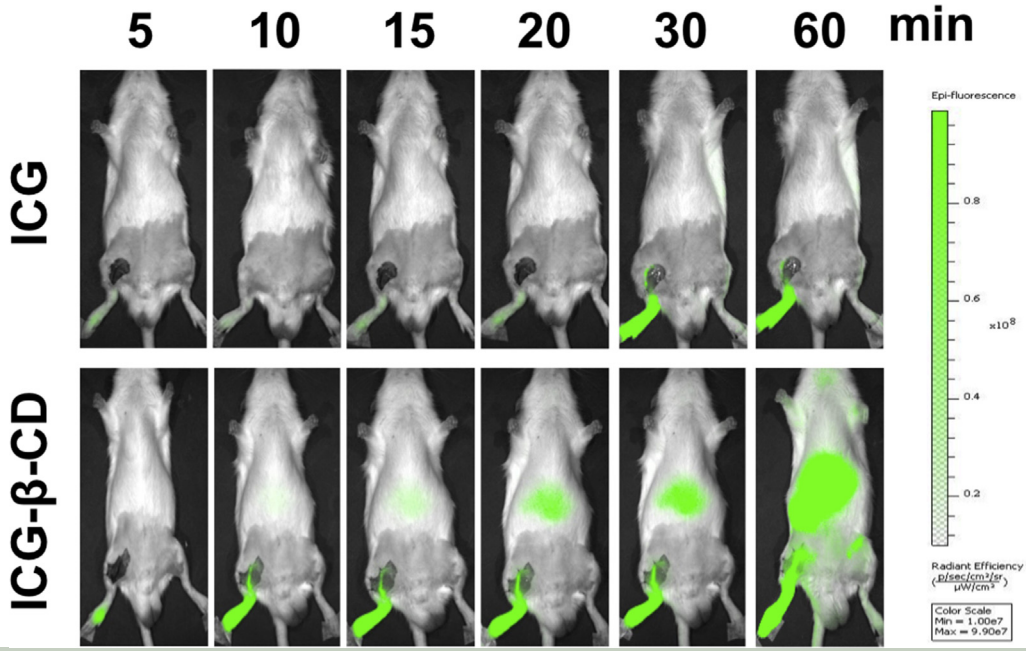

Fig 5. In vivo time-dependent fluorescence imaging of indocyanine green (ICG) and ICG-β-cyclodextrin (ICG-β-CD) at 1.25 mg/mL from 5 minutes to 1 hour after injection using the IVIS Spectrum System.

fluorescence signals of the ICG-β-CD complex could be monitored using clinical NIR fluorescence imaging equipment, suggesting the potential feasibility of the ICG-β-CD complex in clinical microcirculation applications. ICG is a relatively nontoxic fluorescent dye used widely in many clinical fields.[9] When administered to living tissues, ICG can rapidly bind to proteins, such as albumin or lipoproteins, and it becomes fluorescent under NIR light.[9,15,16] However, because of its sulfonyl groups, ICG forms dimers and oligomers in an aqueous solution, making it unstable. These aggregates cause self-quenching of ICG, which reduces its fluorescence in water.[16,17] Thus, we prepared an ICG-β-CD complex assembled from ICG and β-CD in an aqueous state to

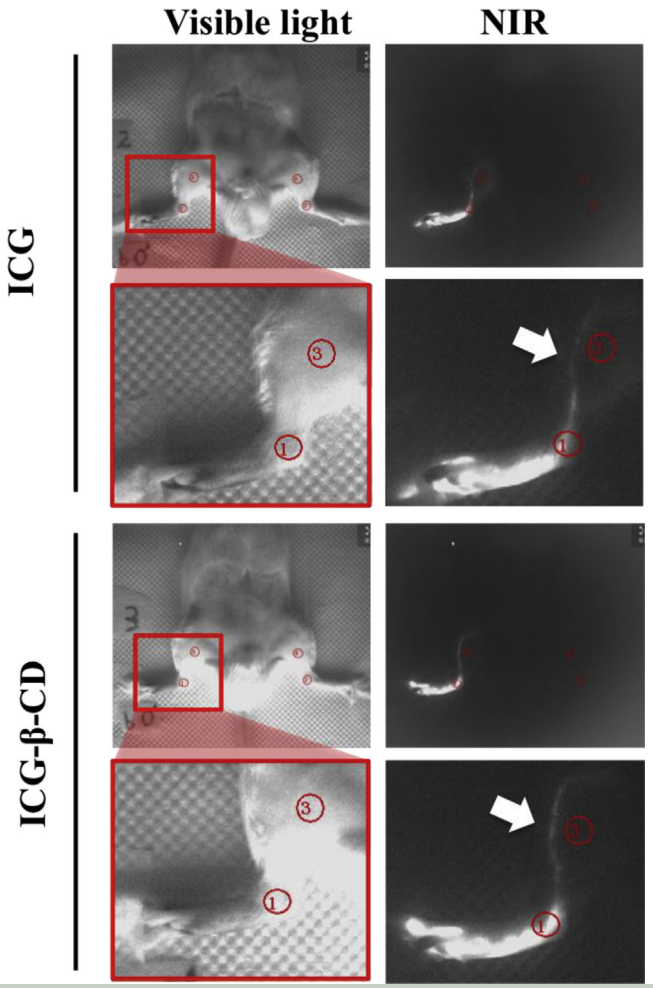

Fig 6. Real-time fluorescence imaging of lymphatic drainage using the DIGI-MIH-001 imaging system at 1 hour after injection. The *red circles* indicate the regions of interest used for calculating the signal background ratio (SBR). *Red circles 1* and *3* indicate the right hindfoot ankle and thigh, respectively. The *white arrows* show the lymphatic vessels. *ICG,* Indocyanine green; *ICG-β-CD,* indocyanine green—β-cyclodextrin; *NIR,* near-infrared.

provide enhanced fluorescence guidance. β-CD can act as a protective cap to prevent aggregation and improve water solubility. Using the host—guest self-assembly process, we successfully prepared the ICG-β-CD complex, which increased its optical intensity. Sevieri et al[17] and Barros et al[18] previously reported the binding between ICG and β-CD in aqueous solutions and determined the aggregation and complexation equilibria of this process.

The use of ICG in experimental studies or the clinical setting can still provide accurate lymphatic function data because, although most ICG will be dimerized and combined with tissues or leaks, increasing the ICG dose can provide sufficient fluorescent molecules to the lymphatic system to allow for lymphatic imaging. Nevertheless, high ICG doses can increase the risk of shock and allergy, and nausea, fever, shock, and other reactions can occur when the preparation has not completely dissolved. Thus, increasing the ICG dose is not an optimal method for improving lymphatic system imaging. Rather, the stability of ICG should be improved. In the early stages after tracer injection, in vivo imaging of the lymphatic drainage pathway of the hind limbs of rats was achieved using the IVIS spectrum system. To further examine the diffusion of the ICG-β-CD complex, we extended the observation time to ≤12 hours and used the clinically available DIGI-MIH-001 system. Theoretically, both ICG and ICG-β-CD will be absorbed into the lymphatic vessels via binding to lipoproteins, which then allows for lymphatic vessel imaging under excitation with NIR light. Speculatively, the increased absorption and transport of ICG-β-CD by the lymphatic system might relate to its molecular structure, with more exposure of positively charged groups and increased binding to negatively charged small molecules.

Our findings suggest that it is feasible to prepare an ICG-β-CD mixture for clinical use. The absorption spectra of ICG-β-CD at 730 nm remained unchanged for 14 days, indicating that ICG-β-CD has good short-term stability (Supplementary Fig). Furthermore, the preparation of ICG-β-CD was quick and convenient, because it only required the addition of sterile water for dissolution when required.

The present study had some limitations. First, although the animal model has been widely used, the pharmaceutical agents can leak into the blood circulation via tiny injury sites during the footpad injection process, even if a small needle has been used. Second, although we observed faster lymphatic transport of ICG-β-CD using both the IVIS and the DIGI-MIH-001 devices and established a convenient method to evaluate the lymphatic diffusion velocity without a skin incision, differences in particle size and particle surface charges can also affect solute movement. Third, the inherent nature of animal experiments means that our results cannot be extrapolated to human clinical treatment. According to the specific molecular structures of ICG and CD, ICG-β-CD is likely to be safe and nontoxic. Nevertheless, more pharmacology studies are required to determine its safety in humans.

CONCLUSIONS

The present study has demonstrated that the fluorescent ICG-β-CD dye of the host and the guest exhibited greater performance than ICG for in vivo NIR imaging in the aqueous state. The lymphatic transport of the ICG-β-CD complex was faster than that of ICG, allowing for more rapid NIR imaging of the lymphatic system. The fluorescence signal of the ICG-β-CD complex could also be evaluated using clinical NIR fluorescence imaging equipment. Therefore, the ICG-β-CD complex is a promising and commercially feasible fluorescent agent

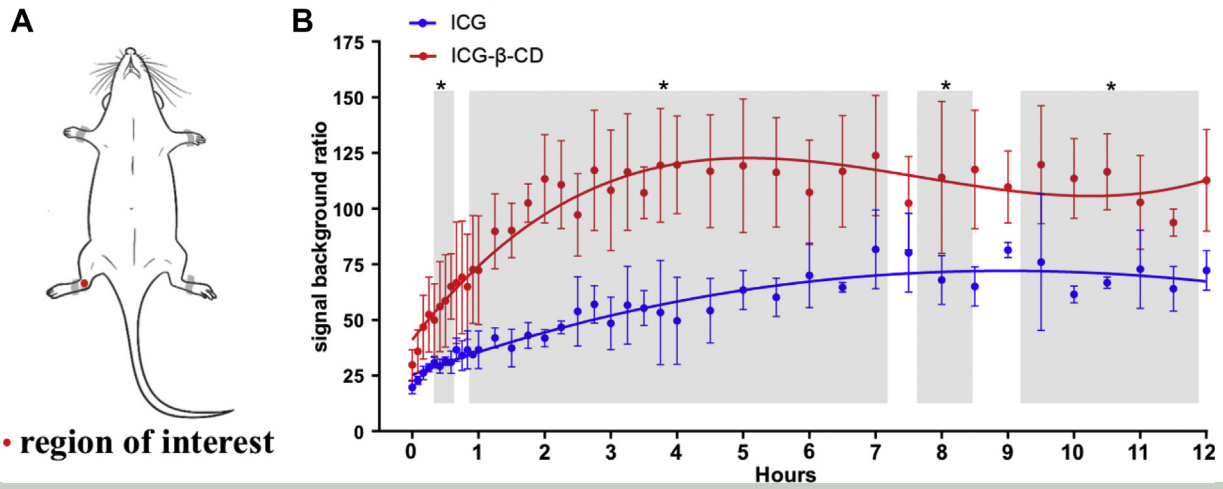

Fig 7. Time-dependent changes in the signal background ratio (SBR) detected in the right hindfoot ankle of rats. Indocyanine green—β-cyclodextrin (ICG-β-CD) showed the fastest diffusion properties. Data presented as the mean ± standard deviation. Two-way analysis of variance with Sidak's multiple comparisons test was performed. The *gray shadow* indicates significant differences between the groups at the indicated time points (*$P < .05$).

for potential use in clinical NIR imaging of the lymphatic system.

AUTHOR CONTRIBUTIONS

Conception and design: FL, WG, DS
Analysis and interpretation: FL, RW, JY, YW
Data collection: JY, MS
Writing the article: FL, YW, DS
Critical revision of the article: FL, RW, JY, MS, WG, DS
Final approval of the article: FL, RW, JY, MS, YW, WG, DS
Statistical analysis: FL
Obtained funding: DS, WG
Overall responsibility: DS
FL and DS contributed equally to this article and share co-first authorship.
DS and WG contributed equally to this article and share co-senior authorship.

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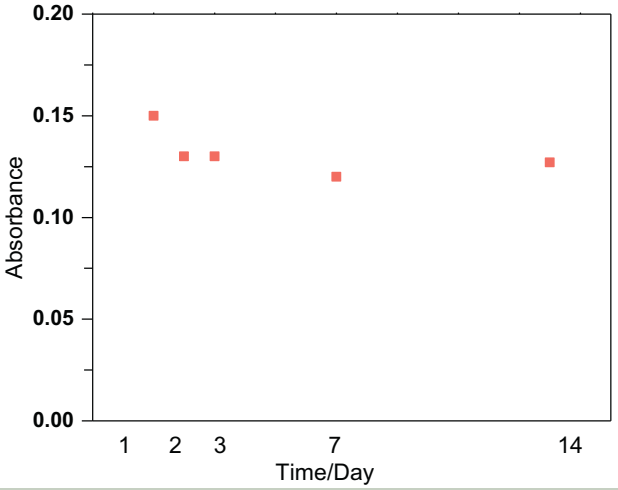

Supplementary Fig. The absorbance spectra of indocyanine green β-cyclodextrin (ICG-β-CD) at 730 nm within 14 days. The absorbance spectra of ICG-β-CD at 730 nm is ~0.15, implying the chemical stability of ICG-β-CD.