

A multimodal approach to assess a liposomal carrier's biodistribution, stability, and clearance

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ABSTRACT

Liposomal carriers, used for site-specific drug delivery, are being investigated for diagnostic approaches by replacing the therapeutic with an imaging contrast agent, exploring potential for selective treatment planning. There remains a critical need to improve *in vivo* assessment of biodistribution, stability, and clearance kinetics of liposomal carriers. This pilot study presents a multimodal approach in which liposome-encapsulated J-aggregated indocyanine green (ICG) dye (Lipo-JICG) is imaged with high spatial resolution using both *in vivo* photoacoustic (PA) imaging, to assess the absorbance characteristics of JICG and monomeric ICG, and *ex vivo* cryofluorescence tomography (CFT), to measure ICG fluorescence. An *in vitro* assay comparing the relationship between absorbance and fluorescence of Lipo-JICG and ICG demonstrated that the absorbance peak shifted from 780 to 895 nm as the Lipo-JICG:ICG ratio increased; meanwhile, the fluorescence decreased drastically as the Lipo-JICG:ICG ratio increased, demonstrating that J-aggregation quenches fluorescence. Twelve mice were then PA imaged pre-injection, then up to 6 days after Lipo-JICG injection. Unmixed Lipo-JICG signal peaked at 30 min post-injection in both liver and spleen; unmixed ICG signal peaked post-injection, decreasing over time in both organs and increasing at 6 days in the spleen. With CFT, ICG fluorescence followed a similar trend, with a maximum at 30 min in liver and at 6 days in spleen, implying that Lipo-JICG continued to break down and excrete through the hepatic system over 6 days post-injection. Future studies will continue to develop this methodology to assess biodistribution, stability, and clearance of liposomal carriers in tumor-bearing murine models.

1. Introduction

Since FDA approval of Doxil® in 1995 [1], liposomes have been widely used as drug delivery vesicles due to their controlled drug release, selective payload delivery to anatomical sites, and reduced concerns for biocompatibility and safety [2–4]. Liposomal carriers are now under investigation preclinically in diagnostic applications wherein the encapsulated therapeutic is replaced with an imaging contrast agent, allowing for personalized treatment planning [5–7]. Additionally, by conjugating a targeting agent to the liposomal surface, it is possible to target them to specific anatomical destinations, allowing for highly selective treatment for diseases with particular molecular profiles.

Despite significant advances with liposomal carriers, there is still a critical need for longitudinal and quantitative assessment of their biodistribution, stability, and clearance kinetics. Recent studies demonstrate biodistribution and clearance assessment of liposomes via radionuclide labeling, facilitating imaging with PET or SPECT [5,8]. Although highly specific, radionuclides expose the patient to ionizing radiation. Additionally, many radiotracers have a relatively short half-life, limiting the time between particle synthesis and imaging and reducing the likelihood of repeated imaging with the same particles [5]. Liposomes can also be labeled with fluorescent dyes and imaged with optical methods; however, these approaches suffer from poor spatial resolution *in vivo*, while their *ex vivo* implementation requires an invasive biopsy that does not provide comprehensive information on spatial distribution [9–12]. Stability of liposomal carriers has been primarily assessed *in vitro*, with limited studies showing assessment of *in vivo*

stability kinetics [13–15].

To assess the complex nature of liposomal biodistribution, we propose a multimodal approach that leverages evaluation of a liposome-encapsulated, J-aggregated indocyanine green (ICG) dye (i.e., Lipo-JICG) with both *in vivo* photoacoustic (PA) imaging and *ex vivo* cryofluorescence tomography (CFT). This single reporter can be assessed with both imaging modalities: J-aggregated ICG (JICG; Fig. 1A) tends to quench fluorescence while causing a redshift and narrowing in the absorbance peak to ~890 nm, allowing for accurate monitoring of agent accumulation with high spatiotemporal resolution using PA imaging [16]. However, if the liposomal shell is compromised, JICG de-aggregates back to its monomeric form (i.e., ICG; Fig. 1A), which results in a drastic increase in fluorescence and a ~100 nm blueshift in absorbance [16], facilitating imaging with CFT. This combined approach allows one to leverage advantages of each modality: PA imaging provides *in vivo*, longitudinal monitoring of Lipo-JICG and ICG, while CFT delivers *ex vivo* ICG distribution with high spatial resolution and a white-light background image for anatomical context. Since ICG signal is associated with degradation of encapsulated JICG, it could serve as a surrogate measure of payload release by liposomal nanocarriers. This pilot study demonstrates the potential of this multimodal approach as a promising preclinical assay to characterize the biodistribution, stability, degradation, and clearance kinetics of Lipo-JICG in a murine model.

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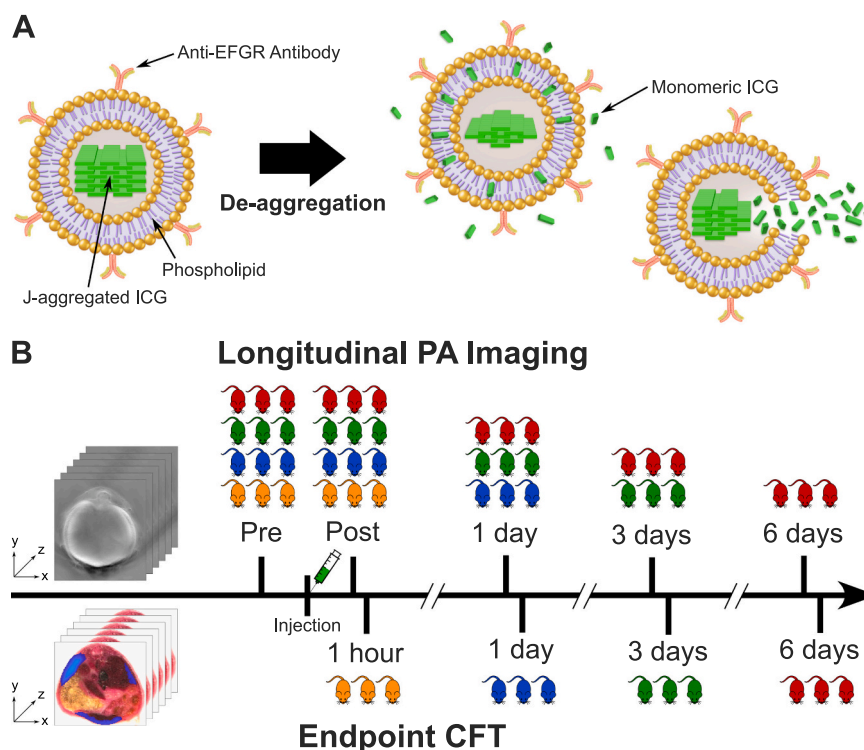


Fig. 1. (A) Schematic of EGFR-Lipo-JICG nanoparticle. JICG de-aggregates into monomeric ICG due to a compromised liposomal shell. (B) Timeline for *in vivo* imaging studies. For all mice, PA imaging was performed pre-injection followed by 30 min of post-injection imaging. Then, 3 mice (i.e., orange cohort) were euthanized for endpoint CFT analysis. PA imaging was repeated for subsequent groups at 1, 3, and 6 days post-injection or until the terminal timepoint was reached for a group (blue = 1 day; green = 3 days; red = 6 days), at which point CFT analysis was performed immediately following PA imaging.

2. Materials and methods

2.1. Lipo-JICG antibody and fluorophore conjugation

Lipo-JICG was obtained (NanoHybrids Inc., Austin, TX) and conjugated, as previously described [16]. Briefly, anti-EGFR monoclonal antibody (ERBITUX, Eli Lilly and Company, Indianapolis, IN) conjugation was performed via maleimide-thiol interaction using a PEG linker to directionally conjugate with the antibody's glycosylated Fc region [17]. Particle size was measured by dynamic light scattering (DLS) before and after conjugation. The resulting conjugates (Fig. 1A) were stored in PBS at 100 OD and 4°C.

2.2. In Vitro validation of Lipo-JICG and ICG absorbance and fluorescence characteristics

To compare the relationship between Lipo-JICG and ICG absorbance and fluorescence, solutions of varying ratios of Lipo-JICG:ICG were prepared ranging from 1.0:0.0 (i.e., 100% Lipo-JICG) to 0.0:1.0 (i.e., 100% ICG; TCI, Portland, OR). Absorbance was measured (Fig. 2A) in a 96-well plate with the Synergy H1 Microplate Reader (BioTek Instruments, Winooski, VT). Fluorescence images (Fig. 2B) of the 96-well plate were then acquired using the Lumina X5 IVIS imaging system (PerkinElmer, Waltham, MA); images were processed with Living Image software (v. 4.8.2; PerkinElmer) and reported as radiant efficiency with a flat-field correction (i.e., for illumination and CCD lens distortion) and cosmic correction (i.e., to account for background light). All measurements were made in triplicate. Line of best fit between Lipo-JICG:ICG ratios versus fluorescence was calculated via linear regression.

2.3. Longitudinal PA imaging protocol

Animal protocols were approved by MD Anderson Cancer Center's

Institutional Animal Care and Use Committee and complied with relevant ethical regulations for animal research. To evaluate *in vivo* Lipo-JICG and ICG localization, PA imaging was performed at multiple timepoints. Twelve athymic NCr nude mice (Taconic Biosciences, Germantown, NY) were randomly assigned into four groups (Fig. 1B; N = 3/group). Mice were imaged on the MSOT inVision 256-TF PA system (iThera Medical, Munich, DE). Multi-wavelength PA images were acquired over z-positions spanning the liver and spleen (parameters in Supplementary Table 1). Mice were imaged twice pre-injection, then EGFR-Lipo-JICG (100 μ L, ~100 OD) was injected via tail-vein catheter; post-injection PA images were acquired for 30 min (N = 12), and at 1 day (N = 9), 3 days (N = 6), and 6 days (N = 3) post-injection (Fig. 1B). 6 days was chosen as the terminal timepoint as it has been previously shown as a typical clearance timeline for similar size liposomes [18]. To help ensure reproducibility, mice were placed in the same position in the scanner for consecutive timepoints.

2.4. PA imaging post-processing

PA images were reconstructed by iterative reconstruction using ViewMSOT software with speed-of-sound corrections applied (v. 4.0, iThera Medical), then imported into MATLAB (v. R2024b; MathWorks, Natick, MA) for further post-processing. First, a signal-to-noise ratio (SNR) mask was generated by calculating the residual between each image pixel and a region that contains only noise, then thresholding on residual values in the noise region [19]. Following SNR mask generation, a wavelength-dependent, surface-fluence correction was applied [16]. Images were unmixed via linear regression for HbO₂, HHb (i.e., to assess blood oxygenation [SO₂]) [20], ICG [21], and Lipo-JICG [16] (spectra shown in Supplementary Figure 1). For quantitative assessment, regions of interest (ROIs) were manually segmented on 800-nm images at each timepoint; this was done blind to previous timepoints to mitigate bias. PA imaging data were analyzed based on volumetric

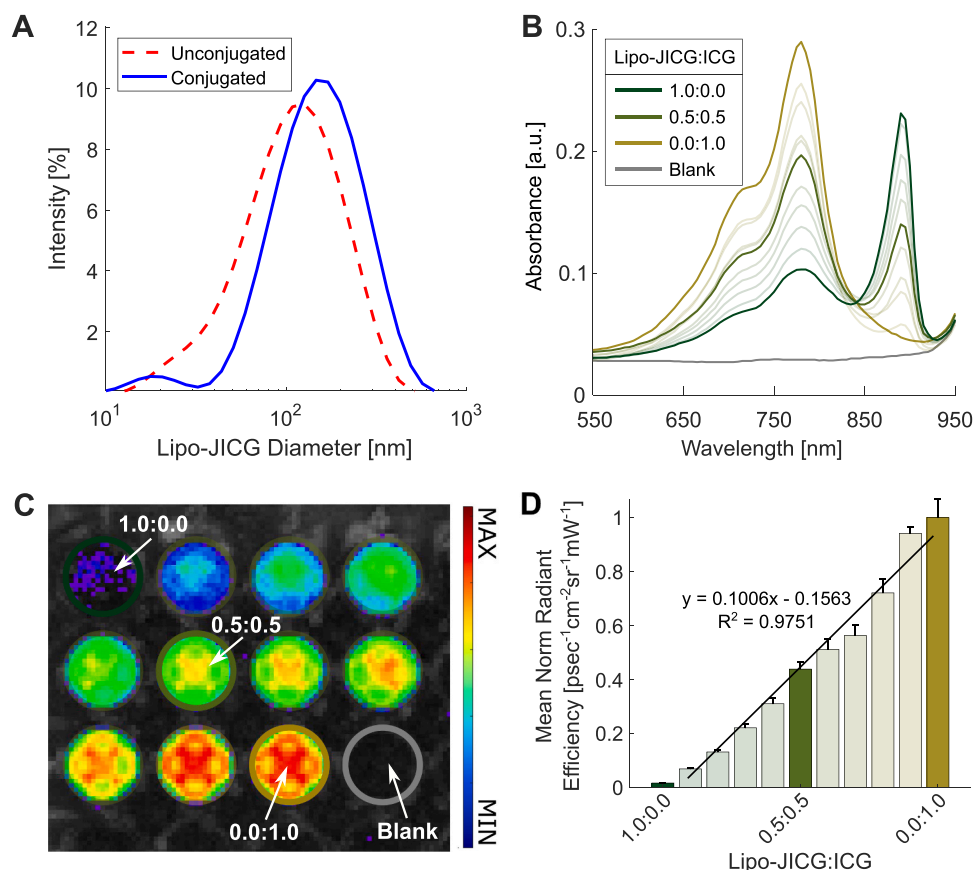


Fig. 2. (A) Lipo-JICG size assessment with DLS shows a mean diameter of 126 nm and 146 nm for unconjugated and EGFR-conjugated particles, respectively. (B-D) Spectral characterization shown as decreasing ratio of Lipo-JICG:ICG. (B) Absorbance spectra demonstrated a blueshift in peak absorbance as the ratio of Lipo-JICG:ICG decreased. (C) Representative fluorescence image of well plates containing Lipo-JICG:ICG ratios (depicted in B & D), from 100 % Lipo-JICG (top left) to 100 % ICG, plus a blank well showing no background fluorescence (bottom right). (D) Normalized radiant efficiency showing increase in fluorescence with increasing monomeric ICG concentration.

mean intensity of each ROI and are compared to their respective pre-injection baseline. SO₂ was calculated and compared pre- to post-injection (i.e., to ensure that no significant changes were observed due to a relatively small injection volume).

2.5. Endpoint cryofluorescence tomography protocol

To evaluate ICG localization and clearance pathways, CFT whole-mouse analysis was performed on matched subjects following PA imaging at multiple endpoints (N = 3/endpoint). An additional group (N = 3) was included 30 min post-injection for a direct comparison with PA data. A naïve control (i.e., no Lipo-JICG injection) was also included for tissue-specific comparison. Mice were euthanized, then flash-frozen and stored until blocked in optimal cutting temperature compound. The block was transferred to the Xerra (EMIT Imaging, South Boston, MA) cryo-chamber, and sequential sectioning and block-face image acquisition cycles were performed for the entire block. White-light and fluorescence images were acquired for each plane with autoexposure setting (parameters in [Supplementary Table 1](#)).

2.6. CFT post-processing

CFT images were reconstructed with EMIT's embedded reconstruction software (EMIT reconstruction tool v1.0). Briefly, fluorescent images were normalized to account for variations in autoexposure acquisition settings. White-light and fluorescent channels were aligned using fiducial markers, and ROIs were manually placed to segment individual mice from the block. Reconstructed images were loaded into

VivoQuant 2020 (Invivo, LLC, Needham, MA) for manual organ ROI segmentation of the liver, spleen, gallbladder, and fecal matter using the white-light channel. Fecal matter was segmented to assess clearance through intestines, which could not be segmented due to the convoluted nature of the tract. Relative fluorescent intensity was analyzed based on volumetric mean intensity of each ROI. One-way ANOVA was used to compare mean ROI intensity across endpoints, and pairwise comparison was completed using Student's *t*-test (MATLAB, $p < 0.05$). Pearson correlation was used to compare PA and CFT results (MATLAB).

3. Results

3.1. In Vitro validation of Lipo-JICG and ICG absorbance and fluorescence characteristics

Optical properties of Lipo-JICG and ICG and the corresponding effect of aggregation on absorbance and fluorescence were characterized in an *in vitro* study. Lipo-JICG peak absorbance was observed at 895 nm, as previously reported [16]; unbound ICG demonstrated peak absorbance at 780 nm (Fig. 2A). A minor, local maximum at 780 nm was observed in the 100 % Lipo-JICG solution due to a charge interaction between PEG (in the liposomal shell) and ICG. The fluorescent signal from this minor interaction was also visible (Fig. 2B), and the relative fluorescent signal increased as the ratio of Lipo-JICG:ICG decreased (Fig. 2C).

3.2. PA imaging analysis of Lipo-JICG and ICG signal

PA imaging data unmixed for Lipo-JICG increased until 30 min post-

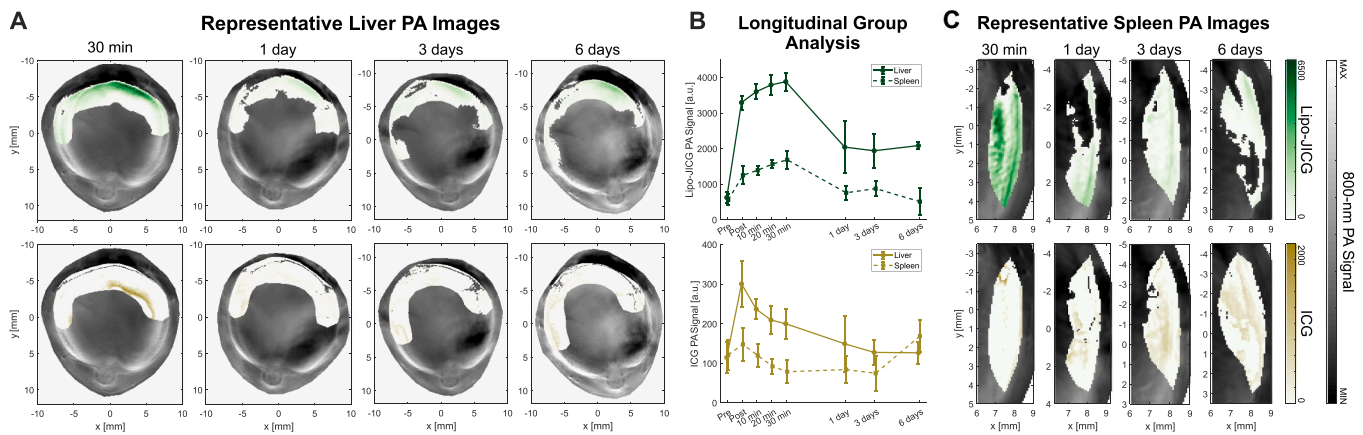


Fig. 3. Representative PA images (800-nm grayscale reference image) in the (A) liver and (C) spleen at 30 min, 1 day, 3 days, and 6 days post-injection. Top row (green) shows unmixed Lipo-JICG signal; bottom row (yellow) is unmixed ICG signal. (B) PA imaging data (mean $\pm$ standard deviation [SD]) unmixed for Lipo-JICG (green, top) and ICG (yellow, bottom) in liver (solid line) and spleen (dashed line) demonstrate a peak in Lipo-JICG signal at 30 min post-injection ($N = 12$), followed by a decrease at later timepoints (1 day $N = 9$, 3 days $N = 6$, 6 days $N = 3$). PA imaging data unmixed for ICG peaks shortly after injection, then gradually decreases over time in the liver; in the spleen, there is an increase at 6 days post-injection, which also correlates with a decrease in Lipo-JICG signal at that time.

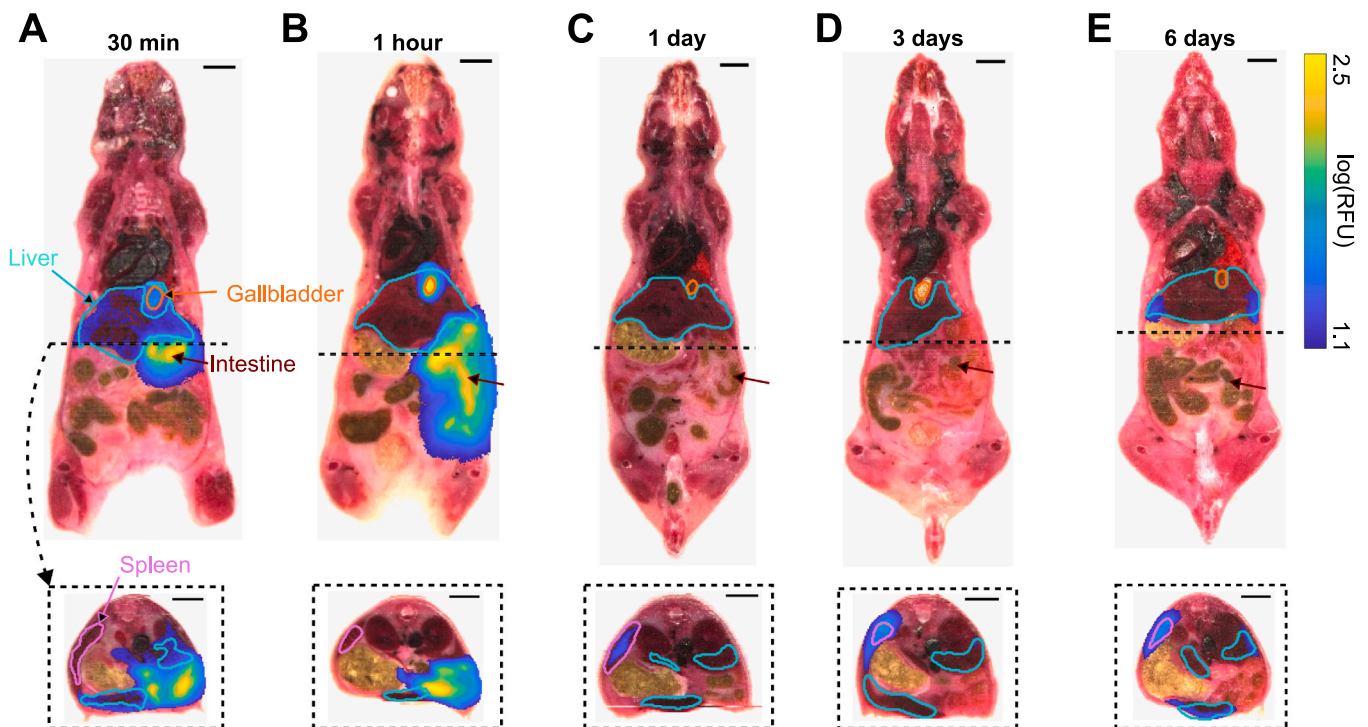


Fig. 4. Representative CFT coronal (top row) and axial (bottom row) images in NCr nude mice of ICG fluorescence overlaid white-light images at (A) 30 min, (B) 1 h, (C) 1 day, (D) 3 days, & (E) 6 days post-injection ($N = 3$ /timepoint). ROIs were manually segmented for the liver (cyan), gallbladder (orange), and spleen (magenta). Intestines are indicated with a maroon arrow, but were unable to be manually segmented due to poor visualization on the images. Black dotted lines on coronal views denote location of corresponding axial plane. Scale bars indicate 5 mm.

injection in both the liver and spleen, while data unmixed for ICG peaked immediately post-injection then decreased for both organs (Fig. 3). At 1 day post-injection, Lipo-JICG signal significantly decreased in both organs, indicating substantial probe clearance. Liver ICG signal remained low through 6 days post-injection; however, spleen ICG signal increased at 6 days. PA imaging data unmixed for SO_2 did not change significantly in either organ pre- to post-injection (Supplementary Figure 2).

3.3. CFT analysis of ICG signal

CFT signal indicated a measurable ICG fluorescence at all timepoints

after Lipo-JICG injection (Fig. 4); the naïve control showed no measurable signal in any organ ROIs. Liver signal at 30 min post-injection presented throughout the organ volume; at 1 h, signal accumulated in bile ducts, gallbladder, and intestines (Supplementary Figure 3). At 1 and 3 days post-injection, liver signal was negligible; however, at 6 days, there was an increase localized to the periphery (Fig. 4E). In the spleen, ICG signal generally increased from 30 min to 6 days post-injection (Fig. 5D). Fecal matter, segmented to assess clearance through intestines, presented with high signal intensity at 1 h post-injection (Supplementary Figure 3F).

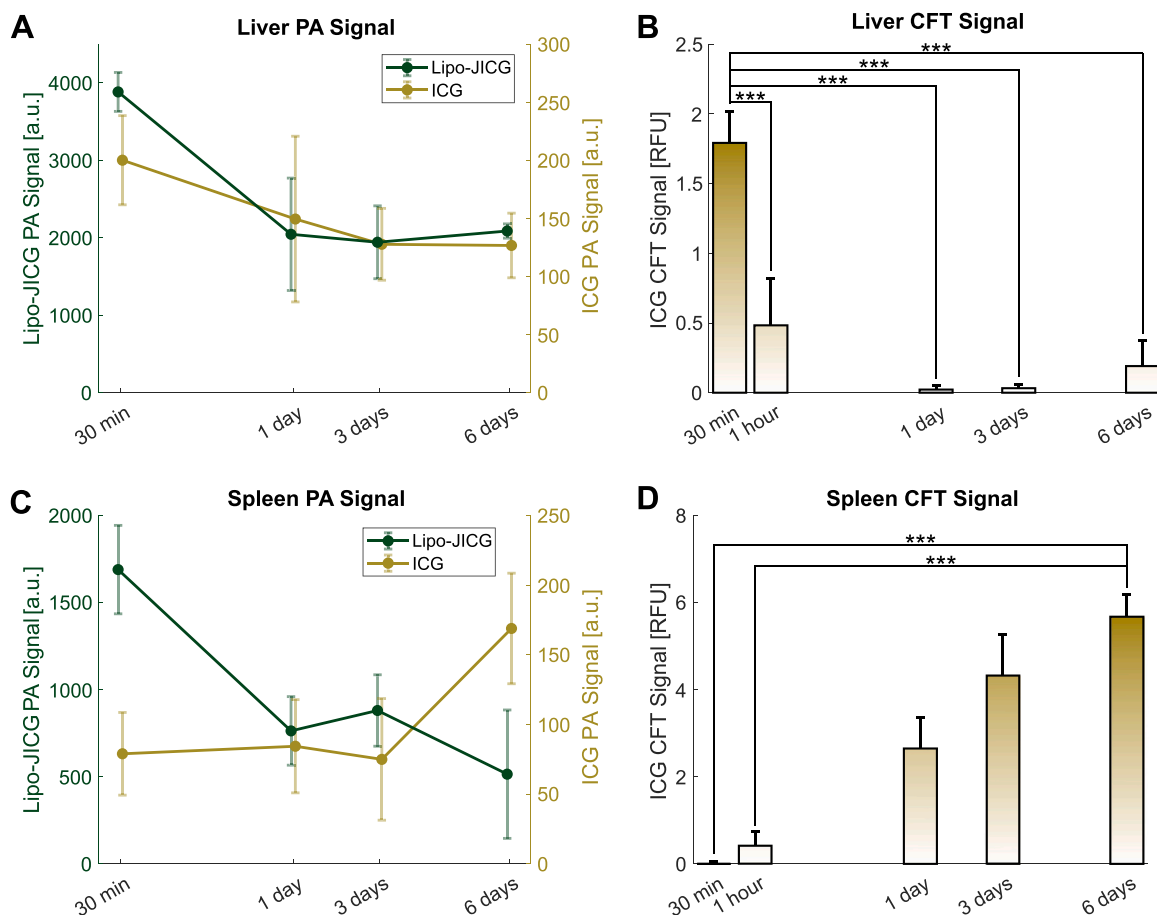


Fig. 5. PA imaging data (mean $\pm$ SD) unmixed for Lipo-JICG (green) and ICG (yellow) in (A) liver and (C) spleen demonstrate a peak in Lipo-JICG signal at 30 min post-injection ($N = 12$), followed by a decrease at later timepoints (1 day, $N = 9$; 3 days, $N = 6$; 6 days, $N = 3$). PA imaging data unmixed for ICG gradually decreases over time in the liver; in the spleen, there is an increase at 6 days post-injection. CFT signal (mean $\pm$ standard error of the mean [SEM]) in the (B) liver and (D) spleen correlate with PA-based ICG signal at the same timepoints ($N = 3$ /timepoint). *** $P < 0.001$.

3.4. Comparison of PA and CFT data

PA and CFT results were compared (Fig. 5) to observe the similarities and differences in *in vivo* absorbance (i.e., PA) and *ex vivo* fluorescence (i.e., CFT) signals. In both liver and spleen, Lipo-JICG absorbance increased until 30 min post-injection, while ICG absorbance and fluorescence were relatively low (i.e., fluorescence below 2 RFU) in both organs through 1 h post-injection. Lipo-JICG absorbance continued to decrease until 6 days post-injection. Over 6 days, both ICG absorbance and fluorescence generally decreased in the liver ($r = 0.71$), while increasing in the spleen ($r = 0.70$).

4. Discussion

Liposomal carriers have gained popularity due to their capacity for targeted delivery without payload degradation and their ability to evade the immune system [22]. The goal of this study was to establish a pre-clinical imaging workflow using PA imaging and CFT to assess biodistribution, stability and clearance pathways of a dye-labeled liposome. The distinct optical absorption and fluorescent properties of JICG and monomeric ICG, as demonstrated in the *in vitro* experiment (Fig. 2), facilitate a multimodal approach for whole-animal monitoring of Lipo-JICG.

Longitudinal PA imaging in a murine model demonstrates a peak in Lipo-JICG signal in the liver and spleen 30 min post-injection, likely indicative of uptake by the reticuloendothelial system (RES) [23]. With an average diameter of 146 nm, EGFR-Lipo-JICG are consistent with

known liposomal clearance dynamics by the RES and often show enhanced permeability and retention effect in targeting tumors [24,25]. Liver ICG signal also peaked within 30 min post-injection, followed by relatively constant Lipo-JICG and ICG signals 1–6 days post-injection. This aligns with previous studies which demonstrated that ICG excretes via the liver to the bile ducts and gallbladder within 30 min of intravenous bolus injection [26]. This is further supported by CFT imaging through 1 h post-injection, where the concentrated signal delineates the pathway from the bile ducts to the gallbladder and intestines. The spleen showed an increase in ICG signal at 6 days post-injection in both PA imaging and CFT; coupled with a slight decrease in Lipo-JICG signal, this is consistent with progressive liposomal shell breakdown and subsequent JICG de-aggregation into monomeric ICG. Although these initial correlations between PA and CFT are promising, one limitation in this study was a relatively small sample size (i.e., $N = 3$ per group). While a small pilot-study sample size was chosen to assess the potential of this approach, future studies will require increased sample sizes to robustly test potential mechanistic claims (e.g., liposomal degradation) supported by PA and CFT data.

The presented multimodal platform could provide longitudinal and semiquantitative biodistribution, stability, and clearance data in future preclinical studies of liposomes or other nanocarriers (e.g., polymerosomes [25,27]). These preclinical metrics also have potential for in-human validation with up-and-coming clinical PA imaging implementation [28–30]. Ligand-targeting liposomes can selectively deliver drugs or contrast agents to an organ that express specific receptors [31]. Future studies will assess biodistribution in tumor models to observe

physiological differences, such as variations in vascularization and perfusion, that may manifest due to the presence of a tumor. Targeted Lipo-JICG formulations will be compared to control formulations to demonstrate the value of specific targeting in such tumor models. Additionally, blood and tissue sampling will be added to future cohorts to further assess the biosafety and compatibility of Lipo-JICG. Another study limitation was the inability to monitor JICG with CFT imaging due to the lack of fluorescence. As a result, direct comparisons between PA and CFT are limited to ICG measurements, which were used as a surrogate measure of JICG degradation. To address this, future studies will attach a secondary fluorescent reporter, whose state is not dependent on JICG degradation, to Lipo-JICG to correlate nanocarrier delivery, degradation, and payload (e.g., ICG dye for Lipo-JICG) release kinetics. This additional fluorophore must be spectrally unique from ICG to avoid crosstalk during CFT analysis as well as loaded at a high enough concentration to get sufficient SNR for CFT imaging.

5. Conclusions

This work presents a multimodal approach, performing complementary imaging of intact and degraded forms of Lipo-JICG, with PA imaging and CFT. Study results demonstrate an application of this method for investigating longitudinal biodistribution, stability, and clearance of Lipo-JICG in a murine model, a critical step for evaluating the safety and efficacy of clinical translation. Future studies will continue to improve upon this platform and investigate other applications where multimodal imaging can provide additional information about exogenous agents and their bioeffects.

CRediT authorship contribution statement

Cayla A Wood: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Claire E Jones:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Ananthakrishnan Soundaram Jeevarathinam:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Riley Watson:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Sangheon Han:** Writing – review & editing, Methodology, Investigation. **Jennifer Meyer:** Writing – review & editing, Investigation. **Konstantin V Sokolov:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Richard R Bouchard:** Writing – review & editing, Visualization, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Konstantin Sokolov reports a relationship with NanoHybrids, Inc. that includes: equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.pacs.2025.100763](https://doi.org/10.1016/j.pacs.2025.100763).

Data availability

Data will be made available on request.

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