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RESEARCH PAPER

Near-Infrared Image-Guided Delivery and Controlled Release Using Optimized Thermosensitive Liposomes

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ABSTRACT

Purpose To engineer optimized near-infrared (NIR) active thermosensitive liposomes to potentially achieve image-guided delivery of chemotherapeutic agents.

Methods Thermosensitive liposomes were surface-coated with either polyethylene glycol or dextran. Differential scanning calorimetry and calcein release studies were conducted to optimize liposomal release, and flow cytometry was employed to determine the *in vitro* macrophage uptake of liposomes. Indocyanine green (ICG) was encapsulated as the NIR dye to evaluate the *in vivo* biodistribution in tumor-bearing mice.

Results The optimized thermosensitive liposome formulation consists of DPPC, SoyPC, and cholesterol in the 100:50:30 molar ratio. Liposomes with dextran and polyethylene glycol demonstrated similar thermal release properties; however *in vitro* macrophage uptake was greater with dextran. Non-invasive *in vivo* NIR imaging showed tumor accumulation of liposomes with both coatings, and *ex vivo* NIR imaging correlated well with actual ICG concentrations in various organs of healthy mice.

Conclusions The optimized thermosensitive liposome formulation demonstrated stability at 37 °C and efficient burst release at 40 and 42 °C. Dextran exhibited potential for application as a surface coating in thermosensitive liposome formulations. *In vivo* studies suggest that liposomal encapsulation of ICG permits reliable, real-time monitoring of liposome biodistribution through non-invasive NIR imaging.

KEY WORDS ICG · image-guided drug delivery · NIR · thermosensitive liposome

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ABBREVIATIONS

DEX	dextran
DPPC	dipalmitoylphosphatidylcholine
DSPE	distearoylphosphatidylethanolamine
ICG	indocyanine green
MPPC	monopalmitoylphosphatidylcholine
NIR	near-infrared
PEG	polyethylene glycol
RES	reticuloendothelial system
Soy-PC	soy-phosphatidylcholine
T_m	transition temperature

INTRODUCTION

"Thermosensitive" liposomes composed of phospholipids that undergo heat activated gel-to-liquid crystalline phase transition slightly above body temperature constitute ideal drug carriers for tissue targets that are accessible to externally-induced hyperthermia. During phase transition, the liposomal contents are subject to rapid release owing to an increase in the vesicle permeability resulting from disordering (or "melting") of the hydrocarbon tails within the liposomal bilayers. Due to their appeal for site-specific release of therapeutic agents, thermosensitive liposome delivery systems have garnered much attention in the area of targeted drug delivery in oncology research (1–5). However, there are some drawbacks to be addressed including: 1) hyperthermia-induced necrosis in surrounding healthy tissue during extended treatment intervals at temperatures at or exceeding 42 °C (6,7), 2) rapid degradation and clearance of liposomes by the reticuloendothelial system which can prevent the drug from ever accumulating at the intended site (8,9), 3) early release of drug content and loss of temperature sensitive membrane components from the

liposomes prior to reaching the site of action, thereby impairing the eventual desired therapeutic effect (10,11), and 4) inter-patient variability in tumor accumulation among different patients/types of cancer and even intra-patient variability during different stages of disease progression, which may ultimately lead to insufficient drug exposure at the tumor site. Ideally, a thermosensitive liposome formulation would be capable of retaining the majority of the drug dose while circulating in the body, yet release sufficient amount in the region of interest to instantly achieve the clinically relevant concentration. With this pursuit in mind, our research approach involves the development of a stable thermosensitive formulation as well as a near-infrared (NIR) liposome monitoring technology. The overall intent of this work is to implement *in vivo* NIR imaging to quantify biodistribution patterns as well as accumulation and release of co-encapsulated chemotherapeutic drugs from the optimized temperature-responsive formulation.

Initially, the major emphasis in this work was placed on developing and optimizing an effective/stable temperature responsive liposome delivery vehicle. It is already well established that the temperature at which the liposome membrane phase transition occurs is related to structural features and interactions of the lipids contained in the bilayer (12). Shorter chain length and more carbon-carbon double bonds will decrease the phase transition temperature (T_m), whereas vesicles with longer, fully saturated lipids tend to have higher T_m. The familiar C_{16} fully-saturated phospholipid, dipalmitoylphosphatidylcholine (DPPC), was chosen as the foundation for our formulations due to its well-established vesicle characteristics and biocompatibility (13). Additionally, DPPC undergoes phase transition slightly above body temperature at approximately 41.5–41.9 °C (14), making it an excellent candidate for hyperthermia-induced liposomal release.

It has also been observed that pure DPPC liposomes have sharper phase transitions than liposomes containing a mixture of lipids, and that permeability at the phase transition can usually be enhanced by incorporating a second lipid into the membrane (14–16). When liposomes are heated to their transition temperature, both gel and liquid-crystalline domains coexist in the membrane thereby creating partially melted boundary regions. Secondary lipids are thought to partition into the grain boundary regions to stabilize liquid crystalline pores in the bilayer (17). Needham *et al.* illustrated that including a small percentage of the lysolipid monopalmitoylphosphatidylcholine (MPPC) enhances permeability and lowers transition temperature compared to pure DPPC vesicles (3,4). However, high water solubility lysolipids, such as MPPC, can potentially desorb from the bilayer or transfer to biological membrane pools *in vivo*, likely resulting in loss of temperature-sensitivity and premature drug release. Other authors have investigated combining DPPC with various alternative lipids, including SoyPC, DSPC, and lyso-palmitoylPC. In this work, we investigated and compared temperature-sensitive formulations of DPPC containing either MPPC or SoyPC as a co-lipid.

In cancer chemotherapy, drug monitoring is often needed to ensure that therapeutic levels of chemotherapy agents are delivered consistently and selectively to the site of action to maximize efficacy and reduce systemic side effects. Naturally, this type of monitoring can be especially important, yet more challenging, for more complex delivery approaches that rely on external triggers (such as thermosensitive liposomes). Recent advances in the area of molecular imaging and nanotechnologies have led to the development of nanocarriers capable of delivering imaging contrast agents and therapeutics concomitantly, thus permitting the study of drug-tumor interaction, biodistribution, as well as pharmacokinetics and pharmacodynamics. Fluorescence-based *in vivo* imaging in the near-infrared (NIR) spectral window has proven particularly beneficial for this purpose. This technique offers a remarkably high signal-to-noise ratio and relatively deep tissue penetration due to the low photon absorption of endogenous biomolecules in the range of 650–1000 nm wavelength (NIR spectrum) (18,19). Because of the inherent benefits of such non-invasive monitoring, we chose to explore use of an NIR fluorophore, indocyanine green (ICG), to facilitate analysis of biodistribution/ tumor accumulation of our liposomal formulations and to explore its potential use in theranostics. ICG is a water soluble NIR dye with molecular weight of 775 Da that has been FDA-approved for visualization of human vasculature in diagnostic applications such as retinal and cardiac angiography (20). ICG itself is rapidly eliminated from the systemic circulation with a half-life of about 3 min (21), however, liposome encapsulation of ICG is presumed to enhance the *in vivo* chemical and photo-stability, and also prolong the circulation half-life. Due to rapid clearance of ICG in its free form, it is expected that NIR signal from ICG would dissipate rapidly once released from the liposomes potentially allowing ICG to serve as an indicator of drug release from liposomes.

This paper encompasses details of our formulation optimization studies as well as evaluation of macrophage uptake, tissue distribution, and comparison between dextran and polyethylene glycol (PEG) liposome surface coatings with the intent to improve the *in vivo* stability of thermosensitive liposomes. Also included in this work is our initial effort at establishing NIR imaging to monitor pharmacokinetics of thermosensitive liposomes. In our data collection process, we examined a sequence of NIR optical measurements for both *ex vivo* and *in vivo* images of tumor-bearing mice. Each set of measurements is used to form an estimate of the liposomal ICG concentration.

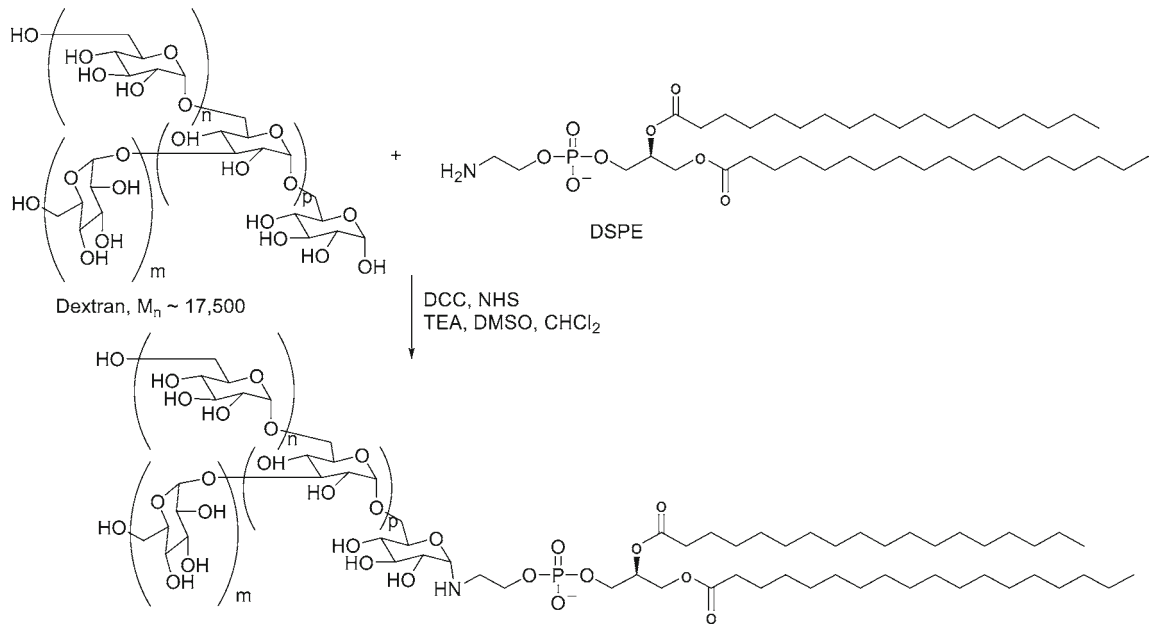
MATERIALS AND METHODS

Synthesis and Characterization of Dextran-DSPE Conjugate

To develop an effective liposome-based NIR imaging system, a stable temperature-sensitive liposome formulation resistant to the reticular endothelial (RES) uptake *in vivo* was sought. To create such a formulation, we tested a non-traditional surface coating, dextran, and evaluated *in vitro* and *in vivo* parameters compared to the more conventional PEG-coated liposomes. Dextran coated liposomes were produced by incorporating a dextran-phospholipid conjugate that was synthesized via a single coupling point with high yield (Scheme 1) (22). In this reaction, 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) was covalently attached to the reducing end of dextran. The reducing anomeric end of dextran differs in reactivity from the numerous hydroxyl groups on the rest of the polymer, essentially providing a selective coupling point. Compared to reductive amination that is usually used to attach a molecule of interest to the reducing end of dextran, the acid-catalyzed condensation is found to effectively link DSPE to dextran with up to 60 % yield. The dextran-DSPE conjugate was a white powder after dialysis and lyophilization. The purity was verified by ^{1}H NMR.

Liposome Preparation

All liposomes were prepared by a standard thin film/extrusion method. Appropriate amounts of phospholipids, cholesterol, and either PEG-conjugated phosphatidylethanolamine (DSPE-PEG2K) or DEX-conjugated phosphatidylethanolamine (DSPE-DEX) were weighted into a round-bottom flask (Table I displays formulation specific ratios). In a typical preparation, approximately 21 mg total phospholipids plus cholesterol were used. Briefly: the weighed components were dissolved in ~2 mL of chloroform/methanol solution (2:1) and rotoevaporated to dryness at 35 °C. The resulting thin film was further dried overnight under in-house vacuum to remove the residual solvent. The following day, the film was hydrated with 1 mL of pre-heated (60 °C) phosphate-buffered saline (PBS), and the flask was maintained at 60 °C for 50 min, vortexing at 10-minute intervals. The resulting vesicle suspension was extruded 19 times through a polycarbonate membrane of 100 nm pore size and 19 mm diameter (Avanti) set at 65 °C using an Avanti mini-extruder. When appropriate, the final concentration of lipids was determined by colorimetric assay for total phosphorous (23). Briefly: an aliquot of the samples were digested with 8.9N sulfuric acid at 210 °C for 25 min, followed by hydrogen peroxide for 30 min at 210 °C. Ammonium molybdate(VI) tetrahydrate and ascorbic acid were added to complex the free phosphorous, the samples were heated at 100 °C for 7 min, then absorbance was read at 820 nm. All calorimetric measurements were performed with a VP-DSC (MicroCal) differential scanning calorimeter interfaced to a computer. The calorimeter consists of two cells, one for the sample and one for reference (containing buffer). All calorimetric scans were performed at a scan rate of 1 K/min.



Scheme I Synthesis of dextran-DSPE conjugate.

Table I Various Liposome Formulations, Their Size, and Transition Temperatures

Formulation	Lipid Composition	Molar Ratio	Avg. Size (nm)	T_m
S_1	DPPC	–	75.2	41.6 °C
S_2	DPPC: DSPE-DEX	90:0.6	77.3	41.5 °C
S_3	DPPC: DSPE-PEG	90:4	70.4	41.7 °C
I	DPPC: MPPC: DSPE-DEX	90:10:0.6	73.5	40.5 °C
II	DPPC: MPPC: DSPE-PEG2K	90:10:4	78.0	41.1 °C
III	DPPC: MPPC: Chol: DSPE-DEX	90:10:20:0.6	76.3	39.8 °C
IV	DPPC: MPPC: Chol: DSPE-PEG2K	90:10:20:4	61.9	40.6 °C
V	DPPC: MPPC: DSPE-DEX	90:20:0.6	84.8	40.4 °C
VI	DPPC: MPPC: DSPE-PEG	90:20:4	33.3	40.8 °C
VII	DPPC: SoyPC: Chol: DSPE-PEG	100:50:30:1	84.9	45.8 °C
VIII	DPPC: SoyPC: Chol: DSPE-DEX	100:50:30:0.15	111.0	44.2 °C
IX	DPPC: SoyPC: DSPE-PEG	100:50:1	77.5	45.1 °C
X	DPPC: SoyPC: DSPE-DEX	100:50:0.15	105.0	44.7 °C
XI	DPPC: SoyPC: DSPE-PEG	100:75:1	77.5	45.8 °C
XII	DPPC: SoyPC: DSPE-DEX	100:75:0.15	68.9	45.5 °C
XIII	DPPC: MPPC: Chol: DSPE-DEX	90:20:5:0.6	53.0	40.2 °C
XIV	DPPC: MPPC: Chol: DSPE-DEX	90:20:20:0.6	89.4	39.1 °C

Abbreviation: DPPC 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine, MPPC 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, DSPE-PEG2K 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol] 2000, Chol Cholesterol, DSPE-DEX 1, 2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-dextran 17,5000, SoyPC L-α-phoaphatidylcholine (hydrogenated-SoyPC)

Dynamic Light Scattering (DLS)

The particle size distribution of the liposomes was determined by dynamic light scattering (Malvern). The translational diffusion coefficient, *D*, was obtained from fits of the intensity correlation function using the Malvern software. In DLS, the translational diffusion coefficient, *D*, is related to the hydrodynamic radius R_h through the Stokes-Einstein relation

$$D = \frac{k_B T}{6\pi n R_h},$$

where k_B is the Boltzmann constant, T is the absolute temperature, and n is the solvent viscosity. DLS was used to measure the size and/or size distribution of the formulations (Table I).

Liposome Release Assay

A fluorescence-based calcein release assay was employed to measure the leakage and/or heat-induced release of the various formulations. When calcein is present at concentrations >50 mM, the fluorescence is negligible with over 95 % self-quenching; therefore, during preparation of liposomes for these release studies, the dried lipid film was hydrated with 1 mL of preheated (60 °C) 50 mM calcein solution in PBS (pH adjusted to 7.45 with 6M NaOH). Following extrusion, the external calcein was replaced with PBS buffer by size exclusion using a Sephadex G-75 packed column. After appropriate dilution, liposome solutions were incubated either in PBS or 1:1 PBS/FBS (fetal bovine serum) at 37, 40 and 42 °C. Then, the released calcein was monitored with a plate reader (BioTek, Synergy HT, Vermont) using 485 nm as excitation and 525 nm as emission detection wavelength. The % release of dye was determined by the formula $[(I_x - I_i)/(I_d - I_i)] * 100$, where I_x is the fluorescence intensity measured at x time, I_i is the signal determined at the initial time point, and I_d is the fluorescence intensity measured after complete lysis of liposomes, which represents 100 % release.

In Vitro Macrophage Uptake

Liposomes were prepared in the same manner as previously described, except that a hydrophobic fluorescent dye, cholesteryl Bodipy FL C12 (0.12 % *w/w vs.* DPPC), was incorporated into the liposome bilayer by addition to the organic phase during thin film formation. In this study, simplified liposome formulations (S_1, S_2, and S_3) were chosen as a model system to directly compare surface coatings isolated from other factors that could influence the measurements (such as marker release from the vesicle). These formulations contained either only DPPC, or DPPC with DSPE-PEG or DSPE-DEX surface coating. After producing the liposomes, free dye was removed by a size exclusion Sephadex G-75 column pre-equilibrated with PBS. RAW264.7 murine macrophage cells were grown in a humidified incubator (5 % CO_2) at 37 °C in 75 cm^2 flasks containing DMEM high glucose media supplemented with 10 % *V/V* FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin. 24 h prior to beginning the experiment, a nearly confluent flask was scraped and cells were counted; 1×10^6 cells were

seeded in a 12-well plate (Costar). The following day, the fluorescently labeled liposomes were added to the 12-well plates containing the adhered RAW264.7 cells in DMEM with 10 % (*V/V*) FBS at a final liposome concentration of 0.42 mg/mL of lipid. The plates were incubated at 37 °C for 4 h, and control wells were also incubated with the same liposome-media solution at 4 °C for 30 min to determine binding under conditions excluding phagocytosis. After incubation, the plates were washed three times with cold PBS, pH 7.4, fixed with PBS containing 4 % (*V/V*) formaldehyde, and harvested in PBS by scraping. The liposome uptake by the RAW264.7 cells was assessed by using an Accuri C6 flow cytometer. Fluorescence from cholesteryl-Bodipy FL C12 was captured at 530 nm with an excitation wavelength of 490 nm. For each sample, 10,000 RAW264.7 cells were gated from dead and other cells on the basis of their volume (forward light scatter) and granularity (side light scatter).

Preparation and Characterization of Liposomal ICG

To create the ICG loaded liposomes, a 1 mg/mL ICG solution in DI water was used as the thin film hydration solution, and the standard preparation and gel purification method was carried out as described in the above sections. Liposomal ICG was quantified by the same validated HPLC assay described in the section "NIR Imaging-based Pharmacokinetic and Biodistribution." The stability of the thermosensitive liposome formulation (VII) was studied by storing the formulation at 4 °C over 4 days, and the liposome size, leakage (by calcein assay), and quantification of liposomal ICG were monitored daily.

NIR Imaging-Based Pharmacokinetics and Biodistribution Studies

A healthy BALB-C mouse model was implemented to evaluate the feasibility of using NIR imaging to study the biodistribution patterns of thermosensitive liposomes in various mouse tissues. All of the animal procedures were pre-approved by the Mercer University Institutional Animal Care and Use Committee (IACUC). *Ex vivo* NIR images of dissected tissues were acquired using Odyssey® infrared imaging system (LI-COR, Nebraska). In order to validate the NIR technique for quantitative use, a more conventional tissue homogenization/ HPLC method was developed and validated to quantify the amount of ICG present in the tissues. At first, tissues were dissected and weighed, diluted with homogenization buffer (100 mM Tris–HCl, 1 mM EDTA, 200 mM NaCl, 0.5 % SDS) at a ratio of 1 mL per 200 mg of tissue, and then mechanically homogenized. Proteins were precipitated from a 400 μL aliquot with acetonitrile, and after centrifugation, the clear supernatant was evaporated to dryness. The resultant residue was reconstituted in 400 μL of DI water/ acetonitrile at 80:20 ratio, vortexed 30 s, then added to an HPLC vial for analysis. The chromatographic system consisted of a Waters Alliance 2690 separation module with a Waters 996 PDA detector set to scan at 780 nm (Milford, MA, USA), and the separation was performed at room temperature on a Luna C18 column (100 mm × 2 mm I.D., particle size 3.0 μm, Phenomenex, Torrance, CA, USA). The separation of ICG was achieved by the optimized gradient mobile phase conditions described in Table II.

Healthy mice (BALB-C, Charles River, MA, 6–8 weeks old) were intravenously (i.v.) injected with serially diluted liposome encapsulated ICG (formulation VII) at five dosing levels. Fifteen minutes post injection, the mice were sacrificed via CO_2 inhalation, and dissections were carried out to obtain tissues samples of various organs. After weighing dissected tissues, *ex vivo* NIR images were acquired with the infrared imaging system at a resolution setting of 169 μm, 3.9 mm focus offset, and L2.0 intensity setting. Then all tissues were further processed and assayed for ICG concentrations using the validated method described above.

In all subsequent biodistribution/tumor accumulation studies, tumors were established on the dorsal side of mice by s.c. injection of 67NR mouse breast cancer cells (5×10^6). During tumor growth, all mice were carefully monitored for general well-being, weight, and tumor volume. Any mice exhibiting weight loss ≥20 % of the initial weight or tumor volume exceeding 1500 mm^3 were euthanized. For this study, mice were administered liposomal ICG (formulation VII and VIII) at a dosage of 2.5 mg/kg by i.v. injection via the tail vein. The previously described HPLC method was used to determine the ICG loading in the liposome dosage solution and ICG dosing concentrations were normalized by appropriate dilutions. *In vivo* signals were monitored with non-invasive NIR imaging at time intervals of 0.25, 0.50, 0.75, 1, 4, 8, and 24 h post-injection. Then, at the 24-hour time point, mice were euthanized, and organs/ tumors were harvested for *ex vivo* imaging.

Table II The Optimized Gradient Mobile Phase Conditions (Total Run Time=9 Min)

Time	%Mobile phase A[a]	%Mobile phase B[b]
0.00	60.0	40.0
2.00	60.0	40.0
2.50	10.0	90.0
3.50	10.0	90.0
4.00	60.0	40.0

[a] 20 mM sodium acetate buffer (pH 5.0)

[b] Acetonitrile

RESULTS AND DISCUSSION

Liposome Release

The results from the calcein-based release studies in both PBS and 50 % FBS/PBS are shown in Fig. 1. During incubation of liposomes in PBS buffer (Fig. 1a), the release of calcein was minimal at 37 °C for all of the Soy PC-containing formulations (7–8 %) and increased by raising the temperature to either 40 °C or 42 °C. By comparison, calcein release from MPPC-containing formulations also increased by raising the temperature, yet the baseline leakage at 37 °C was substantially higher (17–59 %). When a more biorelevant 50 % bovine serum was added to the incubation media replacing plain PBS buffer, Soy PC-containing liposomes showed the same general trend in terms of release (compare Fig. 1a and b). At 42 °C, release is highest in both DEX and PEG-coated formulations. However, an interesting finding was that liposomes with MPPC released very little calcein even at temperature in excess of the lipid phase transition. Also, the 15-minute burst release data (Fig. 2) shows that in general, the Soy PC-containing formulations outperformed the MPPC formulations. In addition, minimal release at 37 °C was observed for the formulations containing cholesterol, but no significant compromise of release efficiency was observed at 40 or 42 °C. Cholesterol is widely thought to help curb *in vivo* permeability of small hydrophilic compounds below the phase transition temperature by reducing the fluidity of the membrane via interaction with the hydrophobic tail of phospholipids. Cholesterol also helps prevent loss of membrane components to high-density lipoproteins in biological milieu by modifying the packing behavior (24). Since no loss of temperature sensitive release was observed for the SoyPC

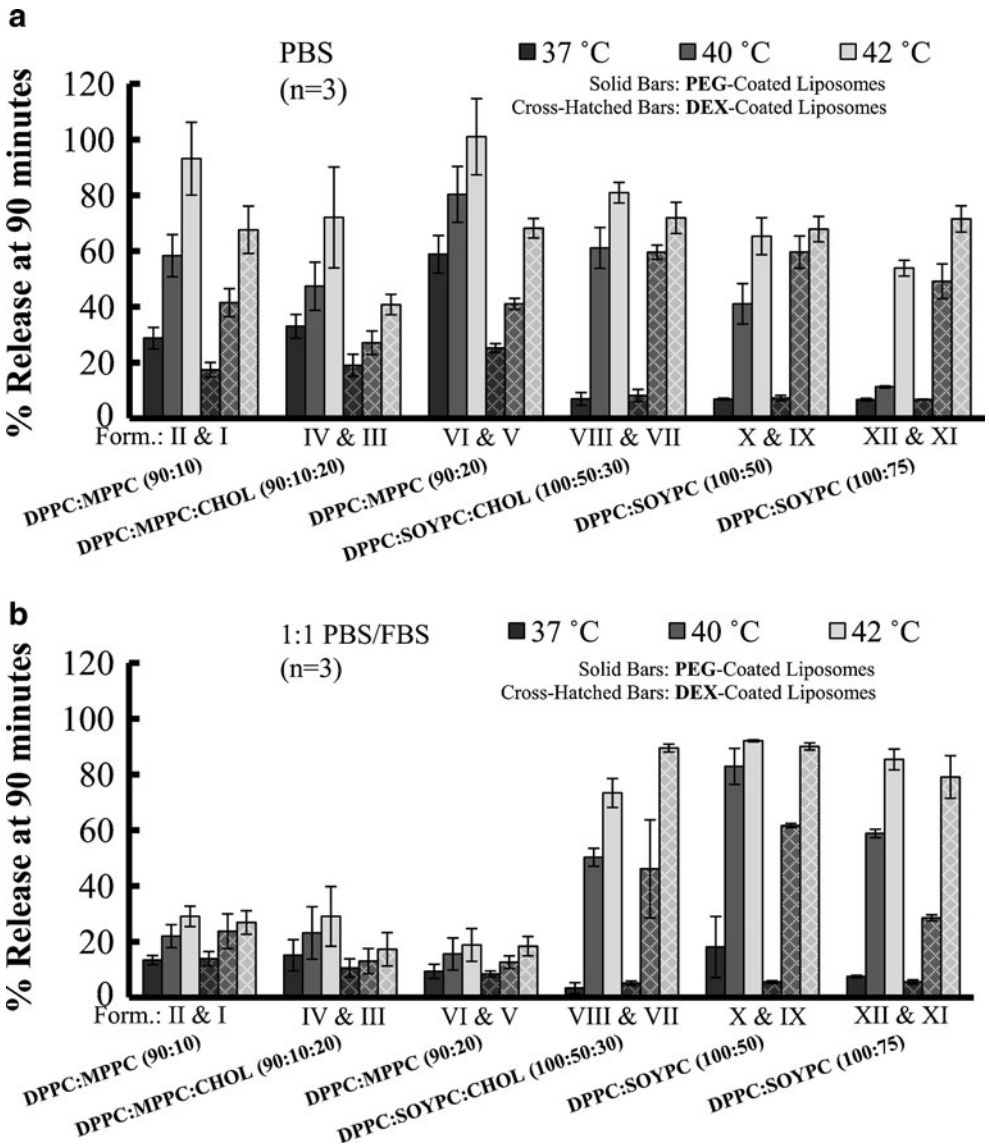


Fig. 1 Release of hydrophilic dye, calcein, from aqueous interior of various liposome formulations measured over 90-minute intervals in extravesicular media of (**a**) phosphate-buffered saline (PBS) and (**b**) 50:50 fetal bovine serum (FBS) / phosphate-buffered saline (PBS) ($n=3$). Each formulation contains DPPC as the primary constituent with varying amounts of co-lipid (MPPC/SoyPC/cholesterol). Solid bars represent data from the PEG-coated version of the formulation and cross-hatched bars represent data from the dextran-coated version.

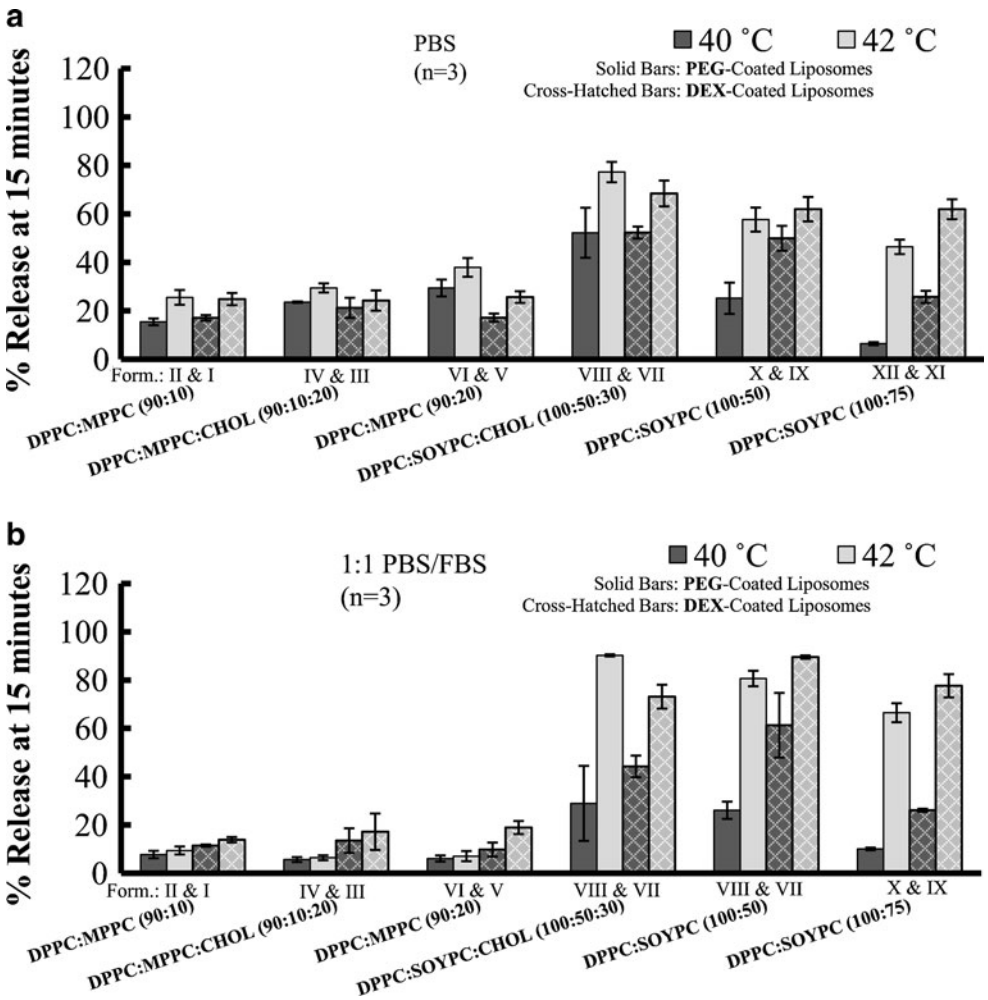


Fig. 2 "Burst" release of hydrophilic dye, calcein, from aqueous interior of various liposome formulations measured over 15-minute interval at 40 and 42 °C in extravesicular media of (**a**) phosphate-buffered saline (PBS) and (**b**) 50:50 fetal bovine serum (FBS) / phosphate-buffered saline (PBS) (n=3).

formulations containing cholesterol, these formulations were prioritized for additional screening.

To supplement the 90 min release studies for formulation VII and VIII, an extended release profile was carried out at 37 °C over 3 day periods using PEG, DEX, and varied hybrid coatings of PEG and DEX (Fig. 3). The data showed substantial release for all formulations, and no clear trend was observed to implicate the two types of surface coating in terms of controlling leakage. Compared to all of the other formulations in these release screening studies, formulation VII and VIII

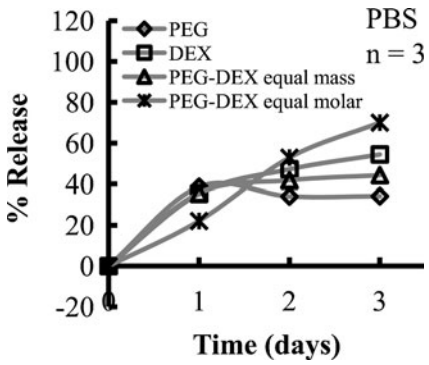


Fig. 3 Surface-coating comparison measured by release of the hydrophilic dye, calcein, from aqueous interior of optimized liposome formulation (DPPC: SoyPC: Chol [100:50:30]) measured over 3-day intervals in PBS (n=3).

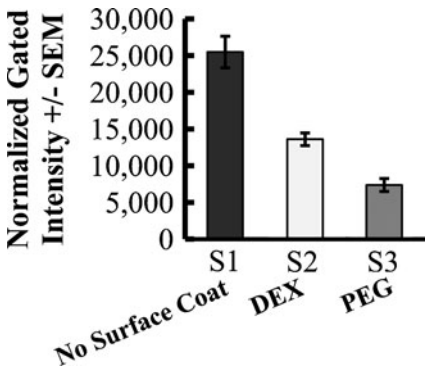


Fig. 4 Macrophage uptake (n=3) of uncoated DPPC liposomes (formulation S1), DPPC liposomes with PEG coating (formulation S3), and DPPC liposomes with DEX coating (formulation S2).

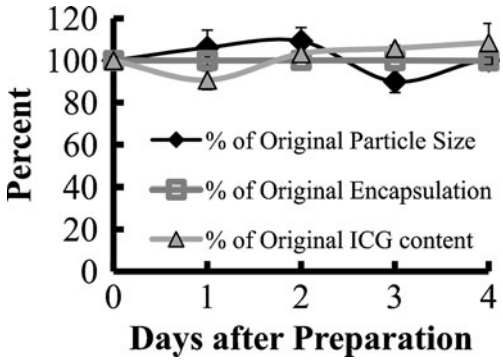


Fig. 5 Storage stability (4 °C) of liposomal (formulation VII): particle size measured by DLS, encapsulation measured by fluorescence monitoring of calcein release, and ICG content assayed by HPLC, all expressed as percent of original (day 0) values (n=3).

demonstrated reasonable stability at 37 °C and favorable release efficiency at 40 and 42 °C, hence; they were subjected to the further studies with the ultimate goal of engineering thermosensitive and NIR active liposomes for anticancer drug delivery.

In Vitro Macrophage Uptake of Liposomes

A major barrier for any nanosized delivery vehicle is avoiding the body's natural defense mechanisms. The majority of liposomes are rapidly eliminated from systemic circulation by macrophages and other phagocytic cells of the RES (25). To overcome this impediment, polyethylene glycol (PEG)-conjugated lipids have been implemented extensively as a liposome surface coating amounting to what are known as "stealth" liposomes (9). One of the drawbacks with these "stealth" PEG-coated liposomes is that the surface coat can potentially induce the production of PEG specific antibodies which can ultimately lead to loss of stability and humoral clearance of liposomes after administering multiple dosages of the formulation (26–29). Of the various potential alternatives (30–35), dextran (DEX) is an example of a naturally occurring, biocompatible polysaccharide consisting of linear (1→6)–D-glucose chains with branches extending mainly from (1→3), and occasionally from (1→4) or (1→2) positions. Due to this extensive branching, DEX polymers with molecular weight greater than 10 kDa have more of a globular morphology compared to that of a non-branching polymer (such as PEG), which we speculated would provide better steric hindrance against phagocytosis relative to PEG coating. Hence, to evaluate this hypothesis, we examined both PEG and DEX-coated versions of the liposome formulations for *in vitro* macrophage uptake.

In agreement with prior studies, the data (n=3) in Fig. 4 shows that plain, uncoated DPPC liposomes exhibit

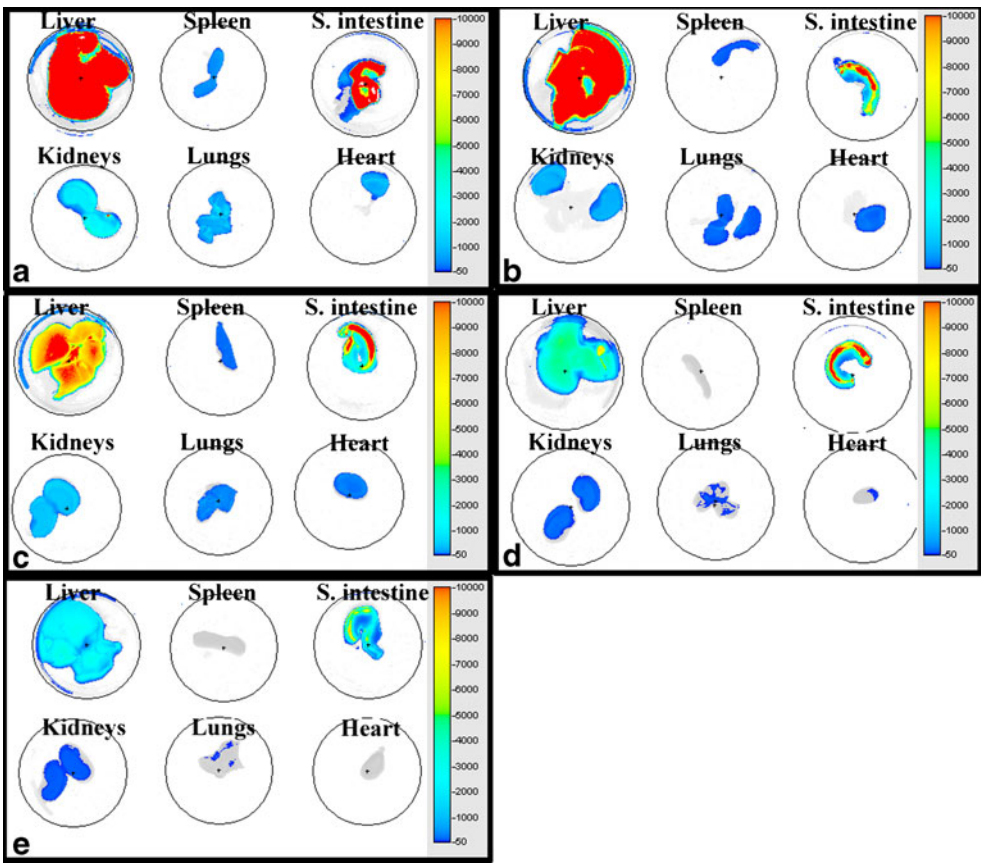


Fig. 6 *Ex vivo* images of dissected tissues (liver, spleen, small intestine, kidneys, lungs, and heart) acquired 15 min post-administration of liposomal ICG (formulation VII) using the Odyssey NIR bioimaging system: (**a**) Stock liposomes, (**b**) 2.5× dilution of stock liposomes, (**c**) 5× dilution of stock liposomes, (**d**) 7.5× dilution of stock liposomes, (**e**) 10× dilution of stock liposomes.

significant macrophage uptake relative to both PEG and DEX surface-coated liposomes which were prepared in a similar fashion. Comparing the two types of surface coating, an elevation in uptake for dextran-coated liposomes was observed compared to PEG-coated. This indicates that the DEX coated liposomes may be more susceptible to binding by serum opsonins that coat the outer membrane and enhance the rate of phagocytosis by macrophage cells. Hence, dextran with an average molecular weight of 17,500 D used in this study may not provide adequate steric hindrance. Future investigations may be extended to other derivatives of dextran to achieve optimal coating.

Storage Stability Evaluation of Liposomal Formulation

To measure short-term storage life of the liposomes, formulation VII was prepared, stored at 4 °C, and subjected to particle size analysis, calcein release assay, and HPLC quantification of ICG content daily for 4 days (n=3 for all measurements). The data from this study, shown in Fig. 5, illustrate good stability in terms of all of these parameters with minimal deviation from original values. Formulations for all of the present studies in this work were prepared fresh either the day of the experiment or 1 day prior.

Quantitative NIR Imaging of Liposomal ICG in Various Organs of Mice

One unique benefit of an NIR imaging approach is that it enables evaluation of the biodistribution of liposomes in multiple organs and tissues (i.e. liver, kidney, spleen, systemic circulation, etc.) with minimal sample preparation. To ensure validity of this method, the absolute fluorophore concentration in mouse tissues was determined using high-performance liquid chromatography (HPLC) as described in the "Materials and Methods" section above. The HPLC method for ICG proved linear up to 2,500 ng/mL (r^2>0.99) with an average recovery of 75.2 % from spiked samples. To establish the quantitation range for NIR image validation, stock gel purified ICG-loaded liposomes (formulation VII) were diluted to 5 different concentrations (1×, 2.5×, 5×, 7.5×, and 10×) and intravenously injected in the lateral tail vein of the mouse. 15 min post-injection, *ex vivo* fluorescence images of dissected tissues (liver, spleen, small intestine, kidneys, lung, and heart) were acquired and then each tissue was further processed and assayed for ICG (Fig. 6). The mass of ICG dye present in each whole organ as determined by

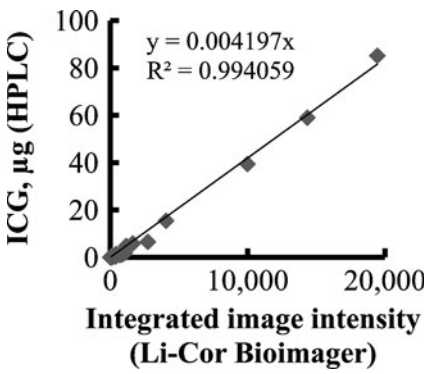


Fig. 7 Mass of ICG dye present in whole organ (HPLC) *vs.* image intensity. This calibration curve was linear over the tested range and was used to determine the absolute mass of ICG present in organs for biodistribution studies.

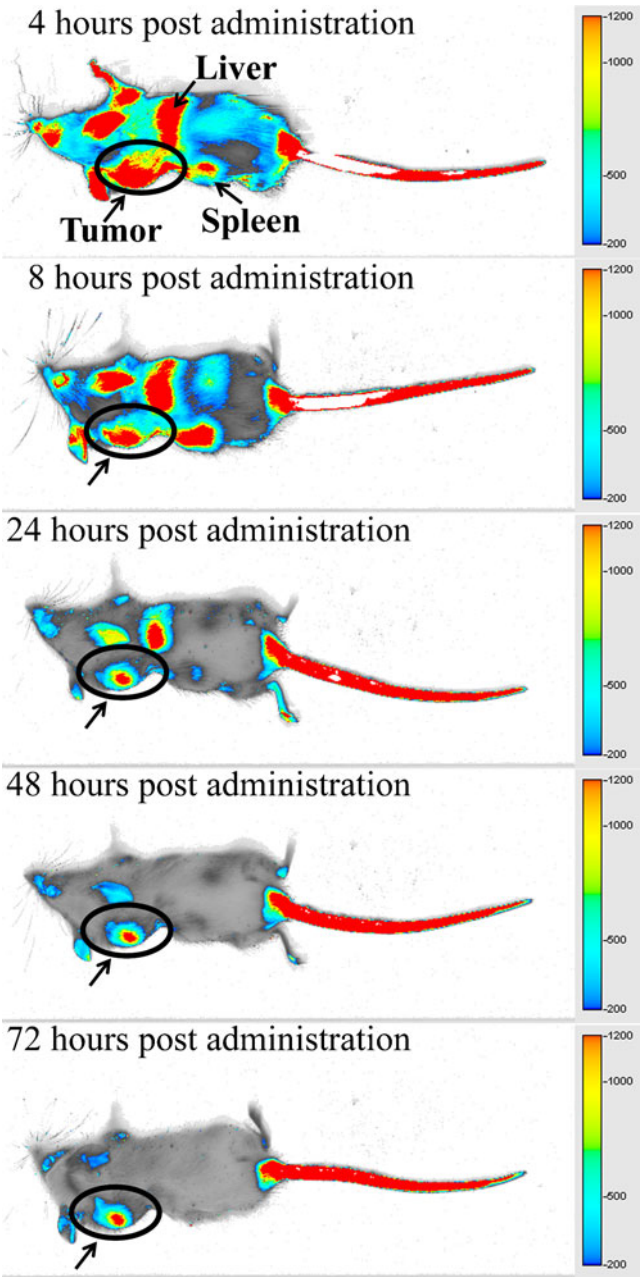


Fig. 8 NIR images of biodistribution and tumor accumulation of liposomal ICG (formulation VII) at various time intervals post dosing.

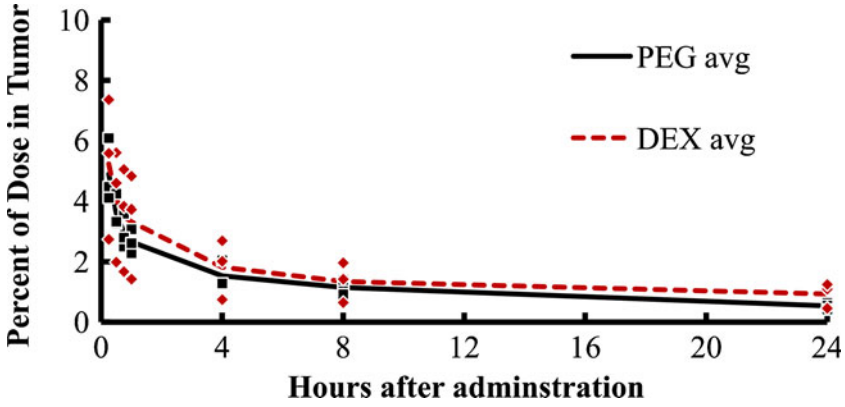


Fig. 9 NIR *in vivo* tumor pharmacokinetic analysis of formulation VII (PEG-coated, $n=3$) and formulation VIII (DEX-coated, $n=3$). Each point represents an individual measurement. The signal decays over time similarly for both formulations. The solid line represents the average of formulation VII, and the dashed line represents the average of formulation VIII.

HPLC analysis correlated well *vs.* image intensity from the NIR bioimager. This calibration curve (Fig. 7) was linear over the tested range and was used to determine the absolute mass of ICG present in organs for subsequent biodistribution studies.

In Vivo NIR Imaging-Based Tumor Accumulation Assessment of Liposomal ICG: A Preliminary Study

For the biodistribution and tumor accumulation study, formulation VII (with PEG coating) was injected intravenously without gel purification and *in vivo* biodistribution was monitored over 72 h. Figure 8 shows significant accumulation of the formulation in the highly vascularized tissues (liver, spleen, and tumor) in the early time-points. After 48 h, the signal dissipated and was detectable primarily in the tumor and injections site. To evaluate organ distribution in the absence of any free dye, this formulation as well as its DEX-coated counterpart (formulation VIII) was administered to separate groups of mice after gel purification. Figure 9 illustrates the *in vivo* monitoring of tumor pharmacokinetics. There was no statistically discernable difference between PEG coated and DEX-coated versions of this formulation in terms of relative tumor concentration levels. After the 24-hour *in vivo* scan, each mouse was sacrificed and *ex vivo* images of various dissected tissues were acquired (Fig. 10). The data from these scans shows similar biodistribution profiles between PEG and DEX-coated versions of the thermosensitive liposome formulation. Both formulations displayed significant accumulation of the formulation in the liver, although the DEX version had a slightly greater accumulation within the liver (corresponding with earlier *in vitro* macrophage uptake data). Tumor accumulation was similar for PEG and DEX formulations.

CONCLUSIONS

We have developed several temperature-sensitive liposome formulations for evaluation of an NIR image-guided drug delivery strategy. Factors such as phase transition temperature, macrophage uptake, and release/stability were investigated, and an optimized liposome formulation was chosen for optical imaging studies. The NIR imaging validation study demonstrated good correlation between the absolute amount of the NIR fluorophore, ICG, and the NIR image intensity, suggesting that NIR imaging is a reliable real-time and non-invasive indicator of liposome biodistribution. Compared with traditional PEG-modified liposomes, DEX-coated liposomes demonstrate potential for application in thermally-sensitive liposomal formulations, however, PEG coating outperformed DEX coating during *in vitro* macrophage uptake studies. Based upon our

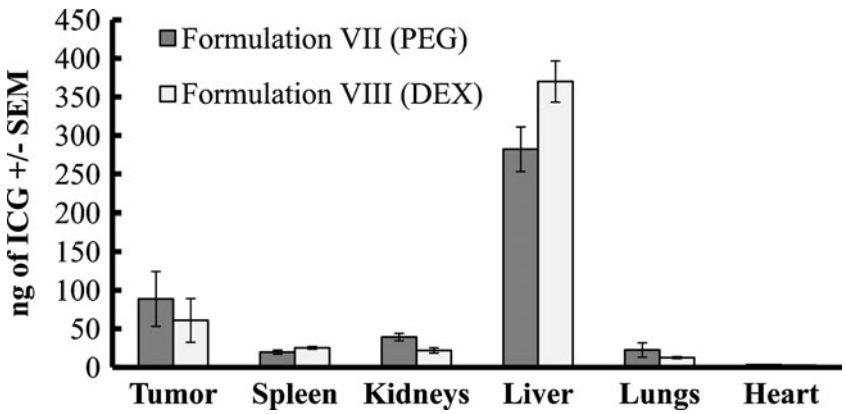


Fig. 10 NIR *ex vivo* analysis of dissected tissues 24 h after dosing of formulation VII (PEG-coated) and formulation VIII (DEX-coated) ($n=3$).

current findings, we anticipate that NIR image-guided drug delivery could prove to be a powerful tool to optimize treatment regimens and monitor tumor drug accumulation with nanoparticle carriers such as liposomes. More extensive and detailed studies, exhibiting improved therapeutic efficacy are required to support the clinical use of this technology.

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