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Tumor Targeting of Functionalized Quantum Dot−Liposome Hybrids by Intravenous Administration

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Abstract: A strategy to target functionalized quantum dot−liposome (*f*-QD-L) hybrid vesicles in the solid tumor tissue of tumor-bearing mice is explored. Functionalized polyethylene glycol (PEG)-lipid coated QD (*f*-QD) were encapsulated into the aqueous core of 100 nm cationic (DOPC:Chol:DOTAP); sterically stabilized, fluid-phase (DOPC:Chol:DSPE-PEG_{2000}); and sterically stabilized, gel-phase (DSPC:Chol:DSPE-PEG_{2000}) liposome vesicles. Double tracking of *f*-QD-L in blood was performed at different time points after intravenous administration in B16F10 melanoma tumor-bearing C57BL6 mice. Cholesteryl [-1-^{14}C] oleate lipids probed the vesicle membrane were followed by liquid scintillation counting while QD were determined independently by elemental (Cd^{2+}) analysis using inductively coupled plasma mass spectrometry (ICP-MS). Rapid blood clearance was observed following intravenous administration of the cationic hybrid vesicles, while incorporation of PEG at the surface of zwitterionic vesicles dramatically prolonged their blood circulation half-life after systemic administration. The "rigid" PEGylated *f*-QD-L (DSPC:Chol:DSPE-PEG_{2000}) hybrid vesicles led to rapid tumor accumulation of peak values (approximately 5% of injected dose per gram tissue) of QD compared to long-circulating *f*-QD that accumulated in the tumor tissue at longer time points. More interestingly, this hybrid vesicle tumor retention persisted for at least 24 h. For almost all types of systems, a preferential cadmium uptake by liver and spleen was obtained. Overall, *f*-QD-L hybrid vesicles offer great potential for tumor imaging applications due to their rapid accumulation and prolonged retention within the tumor. Furthermore, *f*-QD-L offer many opportunities for the development of combinatory therapeutic and imaging (theranostic) modalities by incorporating both drug molecules and QD within the different compartments of a single vesicle.

Keywords: Quantum dots; liposomes; hybrid; biodistribution; tumor targeting; pharmacokinetics; ICP-MS

Introduction

Quantum dots (QD) are explored as fluorescent probes in cell labeling [1−4] and for animal imaging.[5−10] QD were first explored for *ex vivo* imaging by Dubertret et al., who injected polyethylene glycol (PEG)-lipid coated QD into *Xenopus* embryos that were fluorescently labeled without signs of morphological abnormalities.[1] More recently, QD have been used to label zebrafish embryos[11] and chick CAM vasculature,[12] as well as to image living mice and pigs.[5,7,9] They are being explored as imaging agents for the lymph nodes[5,9,13] and organs

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of the reticuloendothelial system (RES) such as liver and spleen.[5,14,15] Decorating the QD surface with hydrophilic polymers (e.g., PEG) was found to reduce the liver and spleen uptake and prolong their blood circulation following systemic administration.[7,16,17]

Previous studies have explored QD targeting to tumors by intravenous administration.[7,16−20] These studies reported low QD tumor accumulation based on passive targeting, however conjugation of targeting ligands, such as antibodies and peptides, onto the QD surface significantly increased tumor accumulation and reduced the QD administered dose and the time required to accumulate. Gao et al. observed minimum tumor accumulation for long circulating, tri-block copolymer-coated QD in a human prostate cancer xenograft model at a dose of 6 nmol after 24 h of injection, while higher tumor targeting was achieved after 2 h of injecting 0.4 nmol of copolymer-coated QD conjugated to prostate-specific membrane antigen (PSMA-QD).[17] In another study, Akerman et al. showed that peptide-coated QD specifically bound to the blood and the lymphatic vessels in an MDA-MB-435 human breast carcinoma xenograft following intravenous administration.[16] Similarly, Cai et al. imaged the tumor vasculature in a U87MG human glioblastoma xenograft using RGD-functionalized QD (RGD-QD).[7] In all these previous studies, the reported tumor targeting was only expressed qualitatively or semiquantitatively. The first quantitative study to evaluate tumor targeting efficiency of QD has been recently published by Cai et al.,[19] reporting a 5-fold increase in the RGD-QD accumulation compared to nontargeted QD (4% ID/g compared to <1% ID/g) using a U87MG human glioblastoma xenograft.

Despite the reported increases in QD tumor accumulation achieved so far, the degree of tumor targeting of QD is still much lower than the tumor accumulation reported for the best sterically stabilized liposome systems with a reported 10−15% of injected dose per gram tissue (ID/g) in the tumor tissue.[21,22] This significant difference in the tumor accumulation between these two types of spherical nanoparticles (QD and liposome) can be attributed to the difference in their size, similar to what has been reported by Ishida et al. for liposomes alone—low tumor accumulation of 60 nm sterically

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stabilized liposomes compared to 100 nm liposomes—indicating that small liposomes can freely pass through the gaps between adjacent endothelial cells.[22] Diagaradjane et al. have recently explained the poor accumulation of QD using a colorectal HCT116 xenograft that expressed high levels of epidermal growth factor receptor (EGFR).[20] They described three phases of QD localization in the tumor: (a) tumor influx (~3 min postinjection); (b) tumor clearance (~60 min postinjection); and (c) tumor accumulation (1−6 h postinjection). Following systemic administration, both targeted EGFR-QD and nontargeted QD rapidly influxed the tumor, however the tumor accumulation phase was only observed with the EGFR-targeted QD. These observations suggested that the increase in tumor fluorescence over time was not due to increased vascular leakage and extravasation into the tumor, but due to EGFR-specific binding and internalization of the targeted QD.

Very recently, we have engineered hybrid vesicles consisting of functionalized (PEG-lipid coated) QD (*f*-QD) encapsulated into the aqueous core of a variety of liposomes.[23] These novel hybrids dramatically improved *f*-QD cell labeling and penetration through 3D-tumor spheroids, without the need of conjugation chemistry on the QD surface. Moreover, *f*-QD-L hybrids successfully labeled solid tumor models *in vivo,* following direct intratumoral injection; however, this procedure is clinically relevant only to superficial and well-localized solid tumors (e.g., head and neck, cerebral carcinomas).

In this work we explored the use of various types of *f*-QD-L hybrid vesicles for *in vivo* systemic solid tumor targeting. Their stability and release profiles in mouse serum were evaluated by turbidimetry and release of vesicle-encapsulated carboxyfluorescein. The blood circulation, tissue and tumor distribution of the different *f*-QD-L hybrid vesicles following intravenous (iv) administration in tumor-bearing mice were determined, in comparison to the more widely used (and commercially available) functionalized PEG-lipid-coated QD. *f*-QD-L hybrid vesicles may constitute a potential combinatory system for the simultaneous delivery of therapeutic and diagnostic agents to a tumor site, and this study is seeking to explore their capacity to accumulate in the tumor tissue by systemic administration.

Experimental Section

Materials. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000} 98%); dioleoylphosphatidylcholine (DOPC 98%); distearoylphosphatidylcholine (DSPC 98%) (Lipoid GmbH, Germany), 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-cholesterol HCl 99%) (Avanti Polar Lipid, USA), green emitting 520 nm, carboxyl functionalized polyethylene glycol-lipid coated QD (QD-PEG-COOH) (Evident Technologies, USA), cholesterol (Chol); chloroform; methanol; Triton X-100; sepharose CL-2B size exclusion chromatography (Sigma, USA), 5, 6-carboxyfluorescein (CF) (Fluka, USA), Vivaspin 4 concentrators MWCO 5000 (Vivascience, Sartorus, Germany), CD-1 mouse serum (Sera Laboratories International, U.K.), dextrose; 30% w/w H_2O_2; sodium dodecyl sulfate (SDS) (Sigma, USA), 75 mm × 10 mm glass culture tube fitted with polyethylene "snap cap"; 50 μL glass capillary tube; Optiphase "safe" scintillation cocktail; isopropanol (Fisher scientific, USA), nitric acid (HNO_3, >69%) (Fluka, USA), 13 mm diameter, 0.45 μm pore poly(vinylidene fluoride) (PVDF) membrane syringe filter (Pall Corporation, USA), ICP-multielement standard solution VI (Merk VI) (Merk, USA), cholesteryl [-1-^{14}C] oleate (specific activity=61 mCi/mmol) (Amersham, U.K.), Beckman tissue solubilizer (BTS-450) (Beckman, USA).

***f*-QD-L Hybrid Vesicle Preparation.** Phospholipids were dissolved in chloroform: methanol (4:1 v/v) mixture. Multilamellar vesicles (MLV) were prepared by evaporating the organic solvent in a 25 mL round-bottom flask using a rotovaporator (BÜCHI, Switzerland), under vacuum for 30 min at 40 °C, then flushed with N_2 stream to remove any residual traces of organic solvent. The dried lipid film was hydrated by addition of 200 μL fractions, with vigorous vortexing, of 0.2 μm filtered 5% dextrose up to a final volume of 1 mL, to achieve a final lipid concentration of 8 mM. Small unilamellar vesicles (SUV) were prepared by further bath sonication (Ultrasonic cleaner, VWR) at 30 °C for 10 min. With high phase transition formulation (DSPC), the film was hydrated at 50 °C for 10 min followed by 15 min sonication. 50 °C was the temperature chosen as it was found to reduce the DSPC liposome size to 100 nm without affecting the QD photostability.

To prepare *f*-QD-L hybrids, the dried lipid film was initially hydrated with 13.7 μL of *f*-QD (COOH-PEG-lipid coated QD, stock concentration 12 μM) suspension, followed by addition of 200 μL fractions, with vigorous vortexing, of 0.2 μm filtered 5% dextrose up to a final volume of 1 mL, then sonicated as described earlier. Final *f*-QD concentration was 1×10^{14} p/mL (160 nM). All prepared samples were flushed with N_2 and left to anneal overnight at room temperature.

***f*-QD-L Hybrid Serum Stability. (A) Turbidmetry.** To evaluate the stability of *f*-QD-L hybrids in physiological conditions, 200 μL (1.6 μmol) of *f*-QD-L hybrids and their corresponding empty liposomes were incubated at 37 °C with 1 mL 50% (v/v) CD-1 mouse serum in phosphate buffered saline (PBS, pH 7.4) to mimic the *in vivo* environment. The interaction of *f*-QD-L hybrids and empty liposomes with the serum proteins was monitored by measuring the changes in the turbidity at 400 nm using a Beckman DU 640 spectrophotometer (Beckman, USA). Relative turbidity was obtained by dividing the sample turbidity in serum by the sample

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turbidity in 5% dextrose, as described elsewhere.[24] Relative turbidity of 1 represents equal turbidity of the sample in serum and in dextrose (i.e., no aggregation).

(B) Carboxyfluorescein (CF) Release. Liposomes and *f*-QD-L hybrids were prepared by lipid film hydration with 200 μL fractions of 0.2 M CF in PBS buffer (pH 7.4) in a stepwise manner up to a final volume of 1 mL. Unentrapped CF was removed using a Sepharose CL-2B size exclusion chromatography column (5 × 1.5 cm). 500 μL of CF-containing vesicles were loaded onto the column and eluted under gravity with 500 μL fractions of PBS buffer. Vesicle-containing fractions were collected and concentrated to the original volume using Vivaspin 4 concentrators (MWCO 5000) and centrifugation at 3000 rpm for 2 h (Eppendrof International, Germany). To study the CF release, 100 μL of CF-loaded vesicles were diluted with 500 μL of 50% v/v CD-1 mouse serum, and incubated at 37 °C with gentle shaking. At different time points, 10 μL samples were withdrawn and further diluted with PBS for fluorescence intensity measurement. Fluorescence measurements were carried out in a quartz cuvette using a PerkinElmer LS-50B luminescence spectrophotometer (PerkinElmer, USA). Samples were excited at 495 nm, and the emission was read at 520 nm, with excitation and emission slit width of 2.5 nm and integration time of 2 s. CF release was studied by monitoring the increase in the fluorescence signal of CF during the release from the vesicles. At high concentrations of encapsulation in vesicles, CF is quenched. As CF is diluted (released to the surrounding medium) self-quenching decreases and fluorescence signals increase. % CF release was determined using the following equation:

$$\% \text{ CF release} = (I_t - I_0)/(I_t - I_0) \times 100\%$$

where I_0 is the initial fluorescence signal, I_t is the fluorescence signal at definite time point and I_∞ is the CF signal after disrupting the vesicles with 10 μL of 10% Triton X-100 solution (in PBS pH 7.4).

Animals and Tumor Models. All animal experiments were performed in compliance with the UK Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures. 8−10 week-old female C57BL6 mice (15−20 g) (Harlan UK Limited, U.K.) were caged in groups of 5 with free access to water. A temperature of 19−22 °C was maintained, with a relative humidity of 45−65%, and a 12 h light/dark cycle. Animals were acclimatized for 7 days before each experiment. To establish syngeneic (B16F10 melanoma) tumor models, mice were inoculated subcutaneously with 1×10^6 B16F10 melanoma cells in a volume of 100 μL of PBS into the right flank using 26G needles. The tumor volume was estimated by measuring three orthogonal diameters (*a*, *b*, and *c*) with calipers; the volume was calculated as $(a \times b \times c) \times 0.5$ mm^3. Biodistribution studies were performed when the tumor volume reached 200−300 mm^3 (7−10 days after tumor inoculation).

Biodistribution of *f*-QD-L Hybrids in Tumor-Bearing Mice. Mice were anesthetized by inhalation of isoflurane and injected via the tail vein with 200 μL of *f*-QD or *f*-QD-L hybrids (equivalent to 1.6 μmol of lipid and 33 pmol of *f*-QD) in 5% dextrose. At different time points the mice were bled by tail vein puncture and 50 μL of blood was collected using a 50 μL capillary tube. Blood withdrawn did not exceed 10% of the mouse blood volume per day. The mice were killed after 4, 24 and 72 h by cervical dislocation. In addition to blood, organs (liver, lung, kidneys, spleen, heart, brain, tumor) were collected and thoroughly washed with distilled water, weighed and analyzed for Cd element.

Quantification of Quantum Dots in Organs. Whole organs were taken (except 150−200 mg of liver) and incubated overnight in 500 μL of lysis buffer (1% SDS, 0.1 M NaCl, 0.05 M Tris, pH 8). The samples were rendered soluble by the addition of 1 mL of 70% nitric acid (HNO_3). All samples were prepared in disposable 10 mm × 7.5 mL glass culture tubes that were fitted with a polyethylene "snap cap". With punctured cap, acid digestion of the samples was carried out by heating the samples in an oil bath at 115°C for 2−3 h for complete tissue solubilization. Later on, the cooled samples were filtered using 0.45 μm pore poly(vinylidene fluoride) membrane syringe filter, to remove any insoluble tissues. The filtrate was rediluted to 5 mL by distilled water and transferred to 15 mL polypropylene centrifuge tubes for storage prior to the measurement. Samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (PerkinElmer SCIEX ICP mass spectrometer, ELAN DRC 6100, USA), as previously described.[15] For all measurements, nitric acid blank, blank tissue samples, spiked samples with known QD concentrations, and Cd standards (Merk Vl) were prepared and tested concurrently with test samples. The total QD concentration in the blood was determined by assuming that the total blood volume was 7.3% of the body weight.[25] The results were expressed as the percentage of injected dose (% ID) per organ or per gram of tissue ($n = 4 \pm$ SD).

Biodistribution of ^{14}C-labeled *f*-QD-L Hybrids in Tumor-Bearing Mice. DSPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2) liposomes and *f*-QD-L hybrids vesicles were radiolabeled with 3 μCi of cholesteryl [-1-^{14}C] oleate (30 μL of 0.1 μCi/ μL stock in toluene) and prepared as described earlier. Mice were anesthetized by inhalation of isoflurane and injected via the tail vein with 0.6 μCi of radiolabeled preparations (equivalent to 1.6 μmol of lipid/200 μL) in 5% dextrose, keeping the lipid and the QD doses similar to the doses used in biodistribution studies analyzed by ICP-MS. At different time points, the mice were bled by the tail vein

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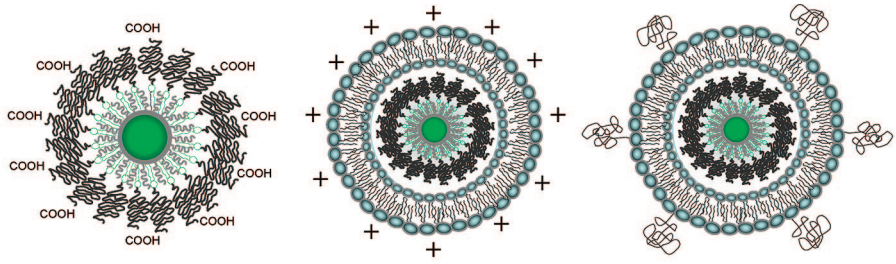

f-QD-L Type	Hydrodynamic Diameter (nm) ±S.D[a]	Polydispersity Index ±S.D[a]	Zeta Potential (mV) ±S.D[a]
f-QD	39±0.00827	0.168 ± 0.013	-7.63 ± 2.02
DOPC:Chol:DOTAP (4:2.5:1)[b]	113±0.867	0.224±0.013	+54.2±2.05
DOPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2)[b]	108±1.25	0.233±0.004	-27.6±1.09
DSPC:Chol:DSPE-PEG_{2000} (1.8:1:0.2)[b]	110±0.551	0.241±0.01	-24.7±0.54

[a] Mean ±standard deviation; n=3
[b] Lipid molar ratio

Figure 1. Schematic diagram showing the structure of *f*-QD (left), and *f*-QD-L hybrid vesicles consisting of cationic (middle) and PEGylated (right) lipids (not drawn to scale). The size, polydispersity index and zeta potential of *f*-QD and *f*-QD-L hybrids used in this study are depicted.

puncture and 50 μL blood was collected using a 50 μL capillary tube. Blood withdrawn did not exceed 10% of the mouse blood volume per day. The mice were killed after 4, 24 and 72 h by cervical dislocation. In addition to blood, organs (liver, lung, kidneys, spleen, heart, brain, and tumor) were collected and thoroughly washed with distilled water, weighed and analyzed for radioactivity by liquid scintillation counting.

Radioactivity Measurement in Organs. Whole organs (except 100 mg of the liver) were transferred to 20 mL scintillation vials. Tissues were solubilized with 1 mL of Beckman tissue solubilizer (BTS-450), shaken overnight at 55 °C, then combined with 20 mL of Optiphase "Safe" scintillation cocktail acidified with 0.7% (v/v) glacial acetic acid to eliminate chemiluminescene, and kept in the dark for 24 h before counting in an LS6500 multipurpose scintillation counter (Beckman, USA). Colored samples (blood and B16F10 tumors) were decolorized with 0.3 mL of 30% H_2O_2 and isopropanol as an antifoaming agent. Samples were shaken at 55 °C for 1−3 h to expel H_2O_2 before adding the scintillation cocktail. The total radioactivity in the blood was determined by assuming the total blood volume was 7.3% of the body weight.[25] The results were expressed as the percentage of injected dose (% ID) per organ or per gram of tissue ($n = 4 \pm$ SD). Radioactivity of the injected dose was $1.4-1.5 \times 10^6$ (dpm).

Statistical Analysis. Data were analyzed using two samples, single sided, Student's *t* test. Differences were considered significant at $p < 0.05$.

Results

Preparation and Characterization of Functionalized Quantum Dot−Liposome Hybrid Nanoparticles (*f*-QD-L). *f*-QD-L hybrids were prepared following the thin lipid film hydration protocol as previously described.[23] Briefly, 8 μmol of phospholipid molecules in chloroform was evaporated, and then the lipid film was hydrated with 160 nM of *f*-QD suspension and bath sonicated, resulting in *f*-QD-L hybrid vesicles of 100 nm in average diameter (Figure 1). The surface charge characteristics of the *f*-QD-L hybrid vesicles were in accordance to the characteristics of the lipid molecules used to form the bilayers. *f*-QD-L hybrids containing cationic (DOTAP) (Figure 1, middle) and PEGylated ($DSPE\text{-}PEG_{2000}$) (Figure 1, right) lipids exhibited zeta potential of +54.2 mV and around −25 mV, respectively (Figure 1). Two types of PEGylated *f*-QD-L hybrid vesicles were prepared, the "fluid" containing DOPC and the "rigid" containing DSPC (see table in Figure 1). The *f*-QD-L hybrid structure was further investigated using confocal laser scanning microscopy (CLSM), agarose gel electrophoresis and atomic force microscopy (AFM). All three techniques confirmed the encapsulation of the *f*-QD inside the liposome core and the absence of surface adsorbed nanoparticles (see Supplementary Figures 1−3 in the Supporting Information), but with some degree of interaction with the lipid bilayer, as previously described.[23]

***f*-QD-L Hybrid Nanoparticle Serum Stability.** To evaluate the stability of *f*-QD-L hybrid vesicles in physiological conditions, 200 μL (1.6 μmol) of empty liposomes

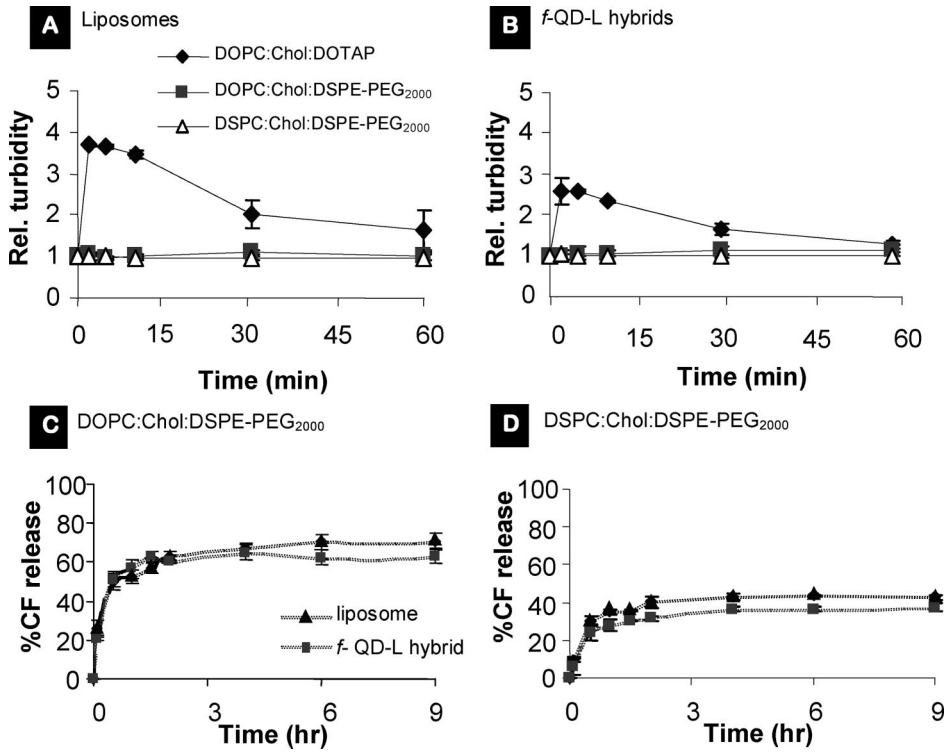

Figure 2. Serum stability of *f*-QD-L hybrid vesicles incubated in 50% mouse serum. Relative turbidity at 400 nm of (A) liposomes and (B) *f*-QD-L hybrid vesicles consisting of cationic (DOPC:Chol:DOTAP), "fluid" PEGylated (DOPC:Chol:DSPE-PEG_{2000}) and "rigid" PEGylated (DSPC:Chol:DSPE-PEG_{2000}) bilayers incubated for 2, 5, 10, 30 and 60 min in 50% mouse serum at 37 °C ($n = 3 \pm$ SD). % CF released from empty liposomes and *f*-QD-L hybrids composed of (C) DOPC:Chol:DSPE-PEG_{2000} and (D) DSPC:Chol:DSPE-PEG_{2000} incubated in 50% mouse serum at 37 °C for 9 h ($n = 3 \pm$ SD).

or *f*-QD-L vesicles prepared in 5% dextrose as described above was incubated with 1 mL of 50% (v/v) CD-1 mouse serum at 37 °C to mimic the *in vivo* environment (approximately half of the volume of complete blood is serum[24]). The effect of serum on empty liposomes and *f*-QD-L vesicles was monitored by measuring the changes in the turbidity. Samples were incubated at 37 °C and analyzed every 2, 5, 10, 30 and 60 min using a Beckman DU 640 spectrophotometer (USA). Turbidity was measured at 400 nm with the corresponding amount of serum alone used as a reference.

Figure 2 depicts the stability of *f*-QD-L hybrid vesicles incubated in 50% mouse serum at 37 °C over time. Turbidity increased sharply for both liposomes (Figure 2A, diamonds) and *f*-QD-L vesicles (Figure 2B, diamonds) of the cationic DOPC:Chol:DOTAP lipid composition, due to interaction with serum proteins, followed by gradual reduction between 10−60 min. Sterically stabilized liposomes and *f*-QD-L vesicles (those containing DSPE-PEG_{2000}) showed no changes in turbidity (Figure 2A and 2B, squares and triangles), which can be explained by the presence of PEG polymer on the vesicle surface (Figure 1) that reduces interactions with serum proteins. Based on these results, only sterically stabilized formulations were used further to determine carboxyfluorescein (CF) release from the vesicles.

In order to evaluate the integrity of the *f*-QD-L vesicles in serum, a high concentration (0.2 M) of the fluorescent aqueous marker CF was encapsulated in the vesicles and free CF was removed by size exclusion chromatography. CF release from empty liposomes and *f*-QD-L hybrid vesicles was studied by monitoring the increase in the fluorescence signal of CF during release from the vesicles. Rapid release was observed from the *f*-QD-L (DOPC:Chol:DSPE-PEG_{2000}) vesicles, that released 30% of encapsulated CF after 5 min and 50−70% between 0.5 and 4 h (Figure 2C, squares). Similar results were obtained for the equivalent liposome system (Figure 2C, triangles), suggesting that the presence of *f*-QD in the vesicle aqueous core did not dramatically change the liposome integrity (that could lead to increased CF leakage), nor did it interact with the encapsulated CF at the molecular level. Modification of the vesicle bilayer by substituting the fluid-phase DOPC bilayer with the "rigid" DSPC (high phase transition lipid exists in gel phase at 37 °C) retarded the CF release from liposomes by a factor of 2, in agreement with previous reports.[26] The % CF release from "rigid" [DSPC:Chol:DSPE-PEG_{2000}] liposome and *f*-QD-L vesicles was 30−40% after 9 h incubation in serum (Figure 2D), indicating that such *f*-QD-L hybrid could offer a platform for systemic multimodal applications.

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***f*-QD-L Hybrid Biodistribution in Tumor-Bearing Mice by Elemental Analysis.** We have previously reported that *f*-QD-L hybrids consisting of cationic and fusogenic lipid bilayers, compared to zwitterionic lipid bilayers, were capable of effectively labeling cancer cells *in vivo* following intratumoral administration.[23] To investigate the behavior of *f*-QD-L hybrid vesicles in living animals further, we examined the blood clearance and tissue biodistribution following intravenous (tail vein) administration of the different *f*-QD-L compared to PEGylated, functionalized QD (*f*-QD) into tumor-bearing C57BL6 mice. The quantitative pharmacokinetic and biodistribution data were generated by elemental analysis (ICP-MS) based on the detection of Cd^{2+} atoms in tissues at different time points, as previously established by others.[15]

Cationic *f*-QD-L hybrid vesicles (DOPC:Chol:DOTAP) exhibited rapid clearance from blood circulation with about 50% and 6% of injected dose (ID) in blood after 5 and 240 min, respectively (Figure 3A, squares). The rapid blood clearance of Cd^{2+} is due to high liver uptake, where approximately 80% ID/g was found 4 h postinjection (Figure 3B, gray bars). Also, 20−30% ID/g was detected in the kidney and spleen (Figure 3B, gray bars).

Previously, studies with sterically stabilized liposomes have reported prolonged blood circulation *in vivo*.[27] Therefore, the effect of steric stabilization on *f*-QD-L hybrid vesicles containing 10 mol% of DSPE-PEG_{2000} was investigated. *f*-QD-L (DOPC:Chol:DSPE-PEG_{2000}) vesicles exhibited up to a 3-fold increase in blood circulation during the first 240 min postinjection (Figure 3A, circles). DOPC:Chol:DSPE-PEG_{2000} *f*-QD-L also showed significantly lower liver uptake, compared to the cationic *f*-QD-L hybrid (45% ID/g and 80% ID/g, respectively) (Figure 3B). Numerous previous studies described that liposomes with bilayers at the gel phase ("rigid" liposomes) circulate longer in the blood.[28] In this study, substitution of DOPC with DSPC to form "rigid" *f*-QD-L (DSPC:Chol:DSPE-PEG_{2000}) vesicles followed by tail vein administration, did not significantly prolong blood circulation of Cd^{2+} (Figure 3A, triangles), resulting in high liver and spleen accumulation even at early time points (4 h postinjection) (Figure 3B, black bars).

The pharmacokinetic behavior of *f*-QD-L hybrid vesicles was also compared to that of commercially available hydrophilic QD (at 33 pmol: same concentration of QD used with *f*-QD-L vesicles) using carboxyl-functionalized polyethylene glycol-lipid coated QD (*f*-QD). The hydrodynamic diameter and surface charge of this material was found to be 39 nm and −7.6 mV (Figure 1), and were injected in tumor-bearing mice without further modification via the tail vein. *f*-QD showed prolonged blood circulation (Figure 3A, diamonds) as 50% ID and 20% ID was detected in the blood after 4 and 24 h, respectively. The tissue biodistribution of *f*-QD was sharply different from the PEGylated *f*-QD-L hybrid vesicles at the early time point (4 h) with about 30% and 10% ID/g found in the liver and spleen (Figure 3B, striped bars). At 24 h postinjection, the *f*-QD concentration in the blood decreased, followed by accumulation primarily in the liver (Figure 3C, striped bars). Similar blood residence and organ uptake data were obtained for "control" *f*-QD sonicated for 15 min at 50 °C, to mimic *f*-QD-L vesicle preparation procedures (data not shown). No significant accumulation of Cd^{2+} in the brain and the heart was obtained with either *f*-QD or *f*-QD-L hybrids over the time course of this study (data not shown), consistent with previous studies using *f*-QD.[15,17] No urine or fecal excretion of Cd^{2+} was observed as confirmed by similar Cd^{2+} levels in the urine and feces for "control" animals (untreated) and the *f*-QD-injected animals (data not shown). In addition, *f*-QD body retention was observed up to 72 h (Figure 3D) with no apparent signs of physiological abnormality or acute toxicity at the doses injected.[15,29,30]

Figure 3E represents the accumulation in B16F10 melanoma tumors of different hybrid vesicle types: cationic *f*-QD-L; "fluid" sterically stabilized *f*-QD-L; and "rigid" sterically stabilized *f*-QD-L compared to the commercially available *f*-QD, after intravenous administration. "Rigid" *f*-QD-L showed a 2-fold increase in tumor accumulation compared to the cationic, the "fluid" and the *f*-QD alone at 4 h postinjection. The "rigid" sterically stabilized *f*-QD-L vesicles reached and accumulated in the tumor raidly (within 4 h) after administration. The *f*-QD alone circulated longer in the blood reaching comparable levels of tumor accumulation (∼5% of ID/g tissue) only after 24 h postinjection.

***f*-QD-L Hybrid Biodistribution in Tumor-Bearing Mice by ^{14}C Liquid Scintillation Counting.** Based on the elemental analysis to determine Cd^{2+} distribution in different tissues, "rigid" sterically stabilized *f*-QD-L hybrid vesicles exhibited short blood circulation times. This was not expected based on previous knowledge of radiolabeled, "rigid" PEGylated liposomes alone of the same lipid composition.[28] In order to investigate this rapid blood clearance profile further, cholesteryl [-^{14}C] oleate was incorporated as a

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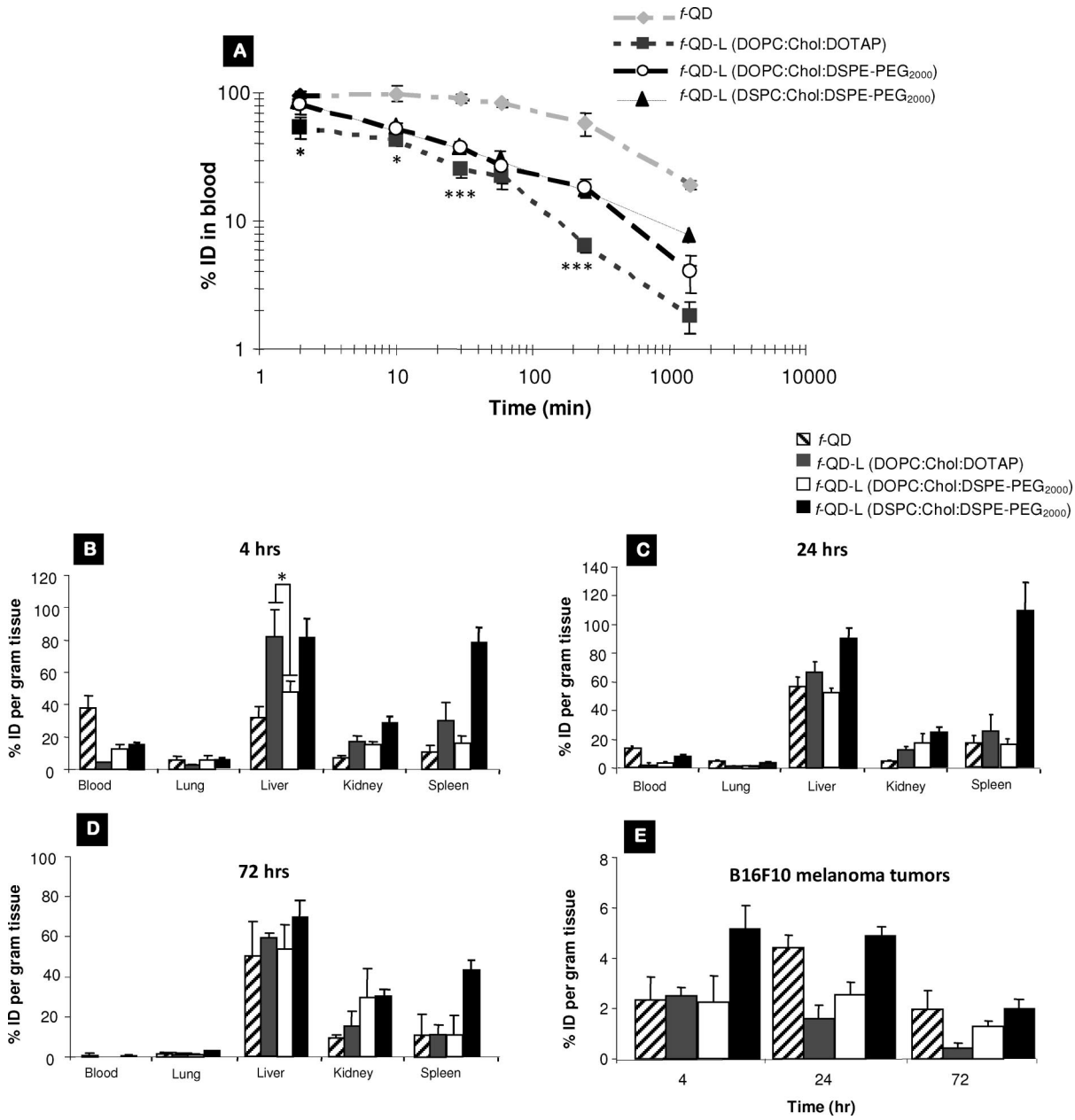

Figure 3. Biodistribution of different QD nanoparticles in tumor-bearing C57BL6 mice after intravenous administration. (A) Blood clearance profile of *f*-QD, cationic *f*-QD-L (DOPC:Chol:DOTAP), "fluid" PEGylated *f*-QD-L (DOPC:Chol:DSPE-PEG_{2000}) and "rigid" PEGylated *f*-QD-L (DSPC:Chol:DSPE-PEG_{2000}) hybrids in tumor-bearing C57BL6 mice after tail vein injection. Tissue biodistribution of above-mentioned nanoparticles at (B) 4 h; (C) 24 h; and (D) 72 h post tail vein injection. (E) Tumor accumulation of *f*-QD and *f*-QD-L hybrids consisting of DOPC:Chol:DOTAP; DOPC:Chol:DSPE-PEG_{2000}; and DSPC:Chol:DSPE-PEG_{2000}) in B16F10 melanoma bearing animals at 4, 24 and 72 h postinjection. QD concentrations in the tissues were determined by ICP-MS ($n = 4 \pm$ SD). Statistical analysis was performed between *f*-QD-L (DOPC:Chol:DOTAP) and *f*-QD-L (DOPC:Chol:DSPE-PEG_{2000}) hybrids, p value was statistically significant *** ($p < 0.001$), * ($p < 0.05$).

nonexchangeable marker for the lipid bilayer [31,32] and ^{14}C-labeled "rigid" PEGylated liposomes and *f*-QD-L hybrid vesicles were injected via the tail vein in tumor-bearing C57BL6 mice. ^{14}C was monitored in blood and organs by liquid scintillation counting.

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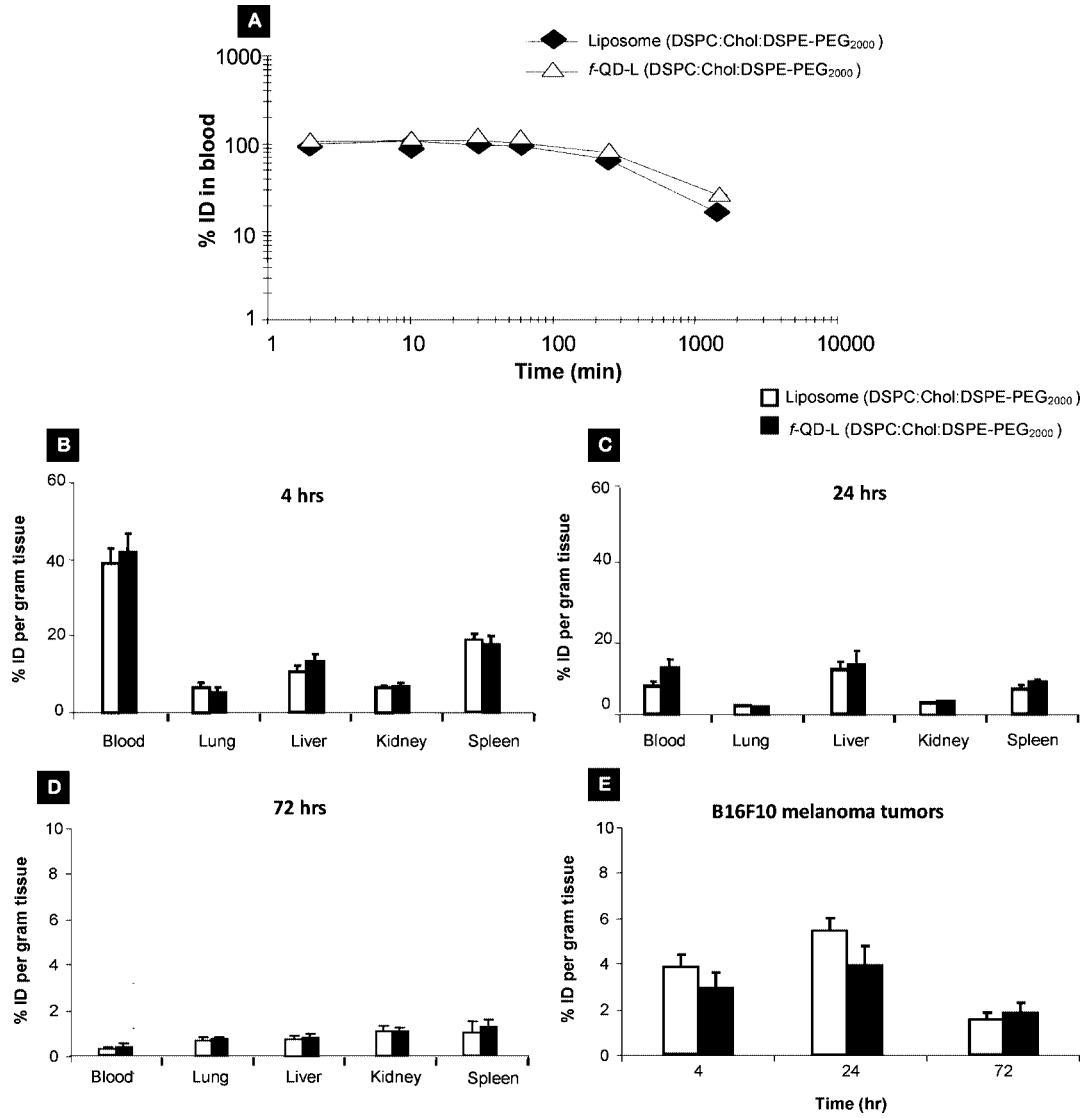

Figure 4. Biodistribution of ^{14}C-labeled *f*-QD-L (DSPC:Chol:DSPE-PEG$_{2000}$) hybrids in tumor-bearing C57BL6 mice after intravenous administration compared to corresponding radiolabeled liposomes. (A) Blood clearance profile of ^{14}C-labeled *f*-QD-L (DSPC:Chol:DSPE-PEG$_{2000}$) hybrids and liposomes after intravenous administration. Tissue biodistribution at (B) 4 h; (C) 24 h; and (D) 72 h post tail vein injection. (E) Tumor accumulation of ^{14}C -labeled *f*-QD-L (DSPC:Chol:DSPE-PEG$_{2000}$) hybrids and corresponding liposomes in B16F10 melanoma tumor 4, 24 and 72 h postinjection. ^{14}C-labeled lipid was analyzed in the organs by liquid scintillation counting ($n = 4 \pm$ SD).

The tissue biodistribution data obtained for *f*-QD-L (Figure 4A, triangles) were similar to empty liposomes (Figure 4A, diamonds), with blood circulation $t_{1/2}$ exceeding 240−300 min, and more than 20% ID detected in blood after 1440 min (24 h). At 4 h postinjection, *f*-QD-L hybrid vesicles (Figure 4B, black bars) and the corresponding empty liposomes (Figure 4B, white bars) both showed low liver, kidney and spleen uptake (10−20% ID/g). At 24 h postinjection, the results were consistent with previous reports[31] revealing a gradual decrease in blood-detected radioactivity[33] (Figure 4C) and at 72 h postinjection less than 2% ID/g tissue was detected in the tissues of all groups (Figure 4D). The time course of tumor accumulation obtained for the ^{14}C-labeled *f*-QD-L hybrids in the B16F10 melanoma model after intravenous administration, compared to the corresponding empty liposomes, is shown in Figure 4E. Similar tumor uptake was observed (~5% ID/g tissue) with both ^{14}C-labeled *f*-QD-L hybrid vesicles and empty liposomes between 4 and 24 h of injection before a gradual decline (to 2% ID/g) after 72 h. Overall, these results indicated that the pharmacokinetic and tissue biodistribution profiles obtained for PEGylated

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f-QD-L compared to liposome alone by radiotracing were almost identical.

Discussion

QD have been investigated for *in vivo* imaging to visualize tumor lesions, the angiogenic endothelium and lymph nodes in living animals.[5,7,10,16,17] Better understanding of the effect of QD structure, surface and elemental consistency on their behavior *in vivo*, such as their blood circulation and organ biodistribution, is warranted to optimize toward clinical translation. Several groups have studied the effect of QD surface coating on their pharmacokinetic behavior.[5,15] Fischer et al., Ballou et al., and Michalet et al. previously observed rapid blood clearance of functionalized non-PEGylated QD and QD conjugated to short polyethylene glycol chains (PEG_{750}), followed by accumulation in the liver and spleen, bone marrow and lymph nodes.[5,14,15] Consistent with findings described in the present study, decorating the QD surface with PEG_{2000} significantly improved their blood circulation (blood half-life ∼4−5 h) (Figure 3A), and reduced their recognition by the liver and spleen (Figure 3B).[7,16,17]

The advantages proposed by the recently engineered *f*-QD-L hybrid systems[23] are (i) versatility of structural and surface characteristics obtained by the selection of different lipid components; (ii) no restriction in the size of QD entrapped in the vesicle aqueous core; (iii) no chemical manipulation of the QD nanocrystal surface. In this study, we constructed different types of *f*-QD-L hybrid vesicles by rational selection of their molecular (lipid) components (Figure 1) and correlated their physicochemical properties and composition with their serum stability (*in vitro*), their tissue biodistribution (*in vivo*), and tumor accumulation (*in vivo*).

Our *in vitro* investigations indicated that *f*-QD-L hybrids of cationic surface character and a fluid-phase bilayer were not stable in serum and released encapsulated carboxyfluorescein rapidly (Figure 2A-C). On the other hand, *f*-QD-L hybrids consisting of a "rigid", gel-phase lipid bilayer (DSPC) and cholesterol exhibited higher serum stability (Figure 2D), which is in agreement with earlier observations regarding the serum stability for liposomes of different compositions.[31,35]

Even though *f*-QD-L hybrids with PEG on their surface exhibited higher serum stability *in vitro* (Figure 2), on systemic (iv) administration all *f*-QD-L types led to rapid blood clearance of Cd^{2+} as determined by elemental analysis (Figure 3). This finding indicated full encapsulation of *f*-QD into the vesicle bilayers leading to a completely different pharmacokinetic profile once compared to, prolonged blood circulation, PEGylated *f*-QD (Figure 3A). Moreover, sharp differences were obtained between the tissue biodistribution of the various *f*-QD-L hybrid types dependent on their lipid composition. Cationic formulations showed faster blood clearance and higher liver uptake compared to fluid-phase, sterically stabilized (DOPC:Chol:DSPE-PEG_{2000}) *f*-QD-L hybrids (Figure 3A,B). No lung accumulation was observed (Figure 3B, gray bars) as may be expected from cationic nanoparticulate systems.[36] This can be explained by the low cationic charge density on the surface of these *f*-QD-L, since the cationic DOTAP lipid constituted only 25% of the vesicle lipid bilayer with zwitterionic DOPC accounting for the rest of the total lipid content. Similar observations have been previously reported by Campbell et al., who reported that increases in the DOTAP molar content from 20 mol% to 50% in liposomes with DOPC led to significant increases in lung accumulation.[37]

Elemental analysis of tissues after injection of "rigid" (DSPC:Chol:DSPE-PEG_{2000}) *f*-QD-L hybrid vesicles indicated the highest liver and spleen uptake compared to other *f*-QD-L types, which requires further investigation. This finding was in contrast to the tissue biodistribution profile in this study for the same hybrid vesicle system obtained by tracking radiolabeled lipid molecules and the corresponding empty liposomes that exhibited low liver and spleen uptake (Figure 4B−D). This discrepancy between ICP-MS (Figure 3B, black bars) and scintillation counting of the liver and spleen (Figure 4B, black bars) may suggest that only a small fraction of the total vesicle population carry *f*-QD and that these *f*-QD-containing vesicles are much more rapidly cleared from the blood and into the liver and spleen compared to empty liposomes. In a recent study, Plassat et al. have highlighted the accelerated blood clearance of PEGylated magnetic-fluid-loaded liposomes (MFL) in mice, following systemic administration.[38] MFL exhibited preferential uptake by the liver and spleen, regardless of their magnetite content. These authors speculated that the presence of magnetite crystals inside the liposomes could induce osmotic effects and result in vesicle swelling that may affect the lipid bilayer elasticity and the PEG conformation on the liposome surface. The observations by the present study and that of Plassat et al. can be further investigated using different PEG-lipid ratios or higher molecular weight PEG and evaluate the conformation of PEG on the *f*-QD-L vesicle surface using small

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neutron scattering,[39] in order to understand the reasons behind the rapid blood clearance of *f*-QD-L hybrids. However, the present findings cannot exclude that the *f*-QD nanoparticles may be avulsed from the liposome core during circulation which requires further evaluation of *f*-QD-L stability in the blood stream.

QD have also been previously explored as fluorescent tools to image solid tumors *in vivo*.[2,7,16,17] In a previous study we reported the direct injection of *f*-QD and a variety of *f*-QD-L hybrids into B16F10 melanoma solid tumor models.[23] In that study, *f*-QD and zwitterionic *f*-QD-L hybrids were shown to be cleared from the tumor in as short a time as 5 min. Contrary to that, cationic *f*-QD-L were retained in the tumor up to 24 h postinjection. However, intratumoral injection is only feasible for superficial and well-localized tumors. The systemic administration route is more clinically relevant to target disseminated (e.g., metastases) and deep-seated tumors, since small, long circulating nanoparticles may interact with tumor cells in the blood circulation. In the preset work, 40 nm PEGylated *f*-QD showed prolonged blood circulation exceeding 4−5 h and tumor accumulation of ~5% ID/g, after 24 h of injection (Figure 3E). "Rigid" *f*-QD-L (DSPC:Chol:DSPE-PEG_{2000}) hybrid vesicles reached equivalent levels of dose accumulation in the tumor, but by exhibiting a very different pharmacokinetic profile. They accumulated in the solid tumor very rapidly and were retained at the tumor site for 24 h as was seen by Cd^{2+} analysis. Importantly, this rapid tumor accumulation and retention was verified in an independent experiment that monitored the (^{14}C labeled) lipid component of the *f*-QD-L hybrid vesicles (Figure 4E).

Based on these findings, *f*-QD-L hybrid vesicles may be useful for systemic tumor imaging, particularly if blockage of liver and spleen can be achieved with conventional empty liposomes to reduce recognition and prolong the hybrid blood circulation and tumor uptake.[36] On the other hand, the rapid blood clearance of the "rigid" *f*-QD-L (DSPC:Chol:DSPE-PEG_{2000}) hybrid vesicles may also be advantageous for tumor imaging applications where a high tumor-to-background ratio is desirable. Gabizon et al. previously reported that little changes in size distribution of liposomes dramatically affect their pharmacological behavior and tumor accumulation *in vivo*.[21] Therefore, we believe that optimizing the *f*-QD-L hybrid mean size distribution can substantially enhance tumor accumulation.[21] It is critically important to obtain an appropriate balance between high tumor uptake and background blood levels of the imaging agent.

In conclusion, in this study we rationally modulated the surface and bilayer characteristics of the recently developed *f*-QD-L hybrid vesicles and correlated them to blood stability (*in vitro*) and tissue biodistribution in tumor-bearing mice, after intravenous administration. Sterically stabilized *f*-QD-L hybrid vesicles have shown high serum stability *in vitro* comparable to corresponding empty liposomes. The *in vivo* profiles obtained for the cationic *f*-QD-L hybrid systems (by monitoring Cd^{2+} atom deposition in blood and tissues) showed faster blood clearance compared to zwitterionic systems. In the case of PEGylated *f*-QD-L, much shorter blood circulation times were observed compared to PEGylated liposomes of the same lipid composition. In terms of tumor passive targeting and retention, rigid PEGylated *f*-QD-L hybrids accumulated in the tumor lesions much more rapidly compared to *f*-QD. Further investigations are warranted to further increase the *f*-QD-L hybrid accumulation in tumors and reduce their uptake in liver and spleen. Moreover, evaluation of *f*-QD-L hybrid systemic toxicity *in vivo* after local and systemic administration should be evaluated before any clinical development. Overall, *f*-QD-L hybrids offer great potential for tumor imaging applications due to their rapid tumor accumulation and can be further adapted easily to construct combinatory therapeutics by encapsulation of *f*-QD and therapeutic agents into a single delivery system.

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Supporting Information Available: Supplementary Figures 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

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