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Ligand Conjugated Low-Density Lipoprotein Nanoparticles for Enhanced Optical Cancer Imaging in Vivo

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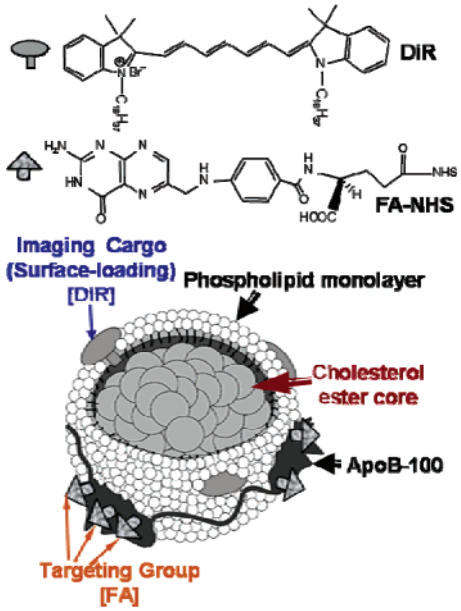
The potential use of low-density lipoproteins (LDL) as nanocarriers for targeted delivery of cancer diagnostics and therapeutics has long been recognized because numerous tumors overexpress LDL receptors (LDLR) to provide the substrates (cholesterol and fatty acids) needed for active membrane synthesis.[1] These naturally occurring nanoparticles have high-payload capacity and are biocompatible, biodegradable, and nonimmunogenic.[1] In addition, the size of LDL particle is precisely controlled (~22 nm) by its apoB-100 component through a network of amphipathic α-helix protein−lipid interactions,[2] setting it apart from liposomes and other lipid emulsions. However, this seemingly perfect delivery platform offers little selectivity over normal tissues since the liver, adrenal, and reproductive organs all express high levels of LDLR[3] and thus would effectively compete for the administered drug or imaging agent. More importantly, the scope for potential LDL based cancer applications is limited by the narrow purview of LDLR-positive tumors. Undoubtedly, broadening the utility of the LDL nanocarrier to a wider range of tumor types would be an important advance for nanomedicine.

We have recently introduced a novel concept of rerouting LDL nanoparticles away from its native receptors to alternate surface receptors or epitopes[4] by conjugating tumor-homing ligands to the exposed active lysine residues in apoB-100, which play a central role in recognition and binding of LDL to LDLR. Proof of this rerouting strategy was demonstrated in vitro as fluorescent labeled folic acid (FA)-conjugated LDL was avidly taken up and accumulated in folate receptor (FR)-overexpressing KB cells while minimal uptake was seen in FR-nonexpressing (CHO and HT1080) cells or LDLR overexpressing cells (HepG2).[4]

Here, we present a ligand-modified, near-infrared (NIR) dye functionalized LDL nanoparticle that not only enables the first in vivo validation of the LDL rerouting principle but also provides the first in vivo application of this concept for enhanced cancer optical imaging in live animals. Thus, this simple and flexible approach could drastically expand the range and increase the accuracy of using LDL particles as nanocarriers for in vivo diagnostic and therapeutic applications.

The methods for preparation of this nanoparticle involve two essential steps: (1) intercalation of a NIR dye, DiR,[5] into the LDL phospholipid monolayer and (2) conjugation of FA to apoB-100 of LDL. Following literature procedures,[4] the desired FA-modified, DiR-labeled LDL nanoparticle, DiR-LDL-FA, was produced at a molar ratio 8:1:105. The synthesis of DiR-LDL-FA is shown in Scheme 1. DiR (ex. 748 nm, em. 782 nm) was selected over the previously used DiI (ex. 549 nm, em. 565 nm) because it allows the noninvasive visualization of in vivo activity of the rerouted LDL particles in living animals.

Scheme 1. Preparation of Rerouted LDL Nanocarrier (Bottom) with FA Targeting Ligand (Middle) and DiR-Based NIR Fluorescent Label (Top)



In vitro confocal microscopy studies were first performed to confirm the FR targeting of DiR-LDL-FA (Figure 1). Following a 4 h incubation period with DiR-LDL-FA (26 μM), a clear and strong pattern of fluorescence was seen throughout the cytoplasm of the KB cells (human epidermoid carcinoma cells, FR^+). Conversely low levels of fluorescence were detected in the HT1080 cells (human fibrosarcoma cells, FR^-) indicating minimal uptake of the DiR-LDL-FA by these cells. The specificity of the FR mediated uptake of DiR-LDL-FA was tested with an inhibition experiment. Figure 1 shows that excess FA completely blocks the uptake DiR-LDL-FA in KB cells as negligible fluorescence was detected. Collectively, the above findings provide strong evidence that DiR-LDL-FA successfully targets FR.

In vivo distribution of DiR-LDL-FA in dual tumor (KB/HT1080) bearing nude mice was monitored with a Xenogen IVIS imager

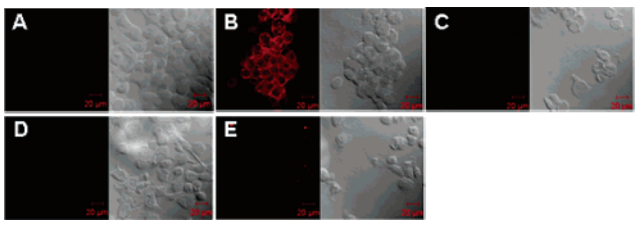


Figure 1. Confocal images of (A) KB (FR^+) alone, (B) KB+DiR-LDL-FA, (C) KB+DiR-LDL-FA+200-fold FA, (D) HT1080 (FR^-) alone, and (E) HT1080+DiR-LDL-FA.

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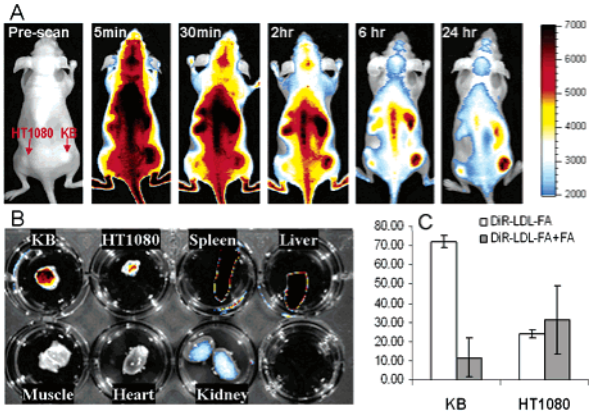

Figure 2. (A) Real time in vivo fluorescence images of KB/HT1080 dual tumor mice with iv injection of DiR-LDL-FA (5.8μM); (B) fluroescence images of tissues and tumors excised at 24 h postinjection; (C) fluorescence readings of tumor extracts from in vivo FA inhibition assay.

(Figure 2A). The selected ICG filter permitted precise detection of the DiR fluorophore as all autofluorescence signals were effectively blocked (Figure 2A, prescan). Immediately following the iv injection, DiR-LDL-FA was rapidly distributed systemically throughout the animal including to the tumor xenografts. By 30 min postinjection regions of high accumulation and retention of DiR-LDL-FA can be seen within the upper abdomen and in the KB tumor. At 2 h the diffuse systemic fluorescence pattern begins to recede (washout effect), and regions of particle retention are clearly defined. Systemic clearance continues over the next 4 h and preferential uptake in the liver and KB tumor become more evident. Finally at 24 h fluorescence is still detected in the liver and surrounding viscera while a strong fluorescence signal is still emitted from the KB tumor. Representative fluorescent images of excised organs and tumor tissues 24 h postinjection of DiR-LDL-FA are presented in Figure 2B. The liver and the spleen displayed saturated levels of fluorescence, indicating avid uptake of this particle by the filtering organs of the reticular endothelial system (RES). In spite of this the efficacy of the FR targeting can still be seen as the KB tumor showed much stronger fluorescence than the HT1080 tumor. The kidneys showed mild levels of fluorescence while that in heart and skeletal muscle was minimal.

The success of this rerouting strategy was further validated in vivo by co-injection of both DiR-LDL-FA and 30-fold free FA as a competitive inhibitor. As shown in Figure 2C, the fluorescence reading of extracts of resected tumors at 24 h postinjection shows strikingly different patterns between the two groups of mice (N = 3). The fluorescence of KB extracts from FA treated mice was 6-fold less than that of their untreated counterparts. The individual fluorescence measures of HT1080 extracts varied considerably in the FA treated group, but on average it was only marginally greater (1.3-fold) than that from the untreated mice.

While convincing evidence of the LDL rerouting strategy was previously demonstrated in cells[4] the behavior of rerouted LDL particles in whole animals may be drastically different as numerous biological barriers and processes in whole living systems can compromise the performance of this nanocarrier. Using a proven FA/FR model system (the high affinity of FA for FR and the high capacity of this system to accommodate large conjugates),[6] the present study examines the in vivo behavior of FA-conjugated LDL particles by NIR fluorescence imaging. Our results confirmed that DiR-LDL-FA was able to selectively target FR expressing tumors, thus effectively validating the LDL rerouting strategy in vivo. However, our biodistribution findings do not support the known pattern of FR expression in normal tissues.[6] The low uptake of DiR-LDL-FA in kidney, heart, and muscle may be explained by the fact that (1) FR present in normal tissues are localized primarily toward the apical surface of polarized epithelia, hence they are inaccessible to intravenous administered particles[7] and (2) the particle size (~25 nm) exceeds the threshold of renal glomerular filtration thus hampering large deposits of the nanoparticle in kidney. On the other hand, the high liver and spleen uptake of DiR-LDL-FA likely involves competitive uptake processes by the RES. Interactions with circulating apoproteins present yet another means whereby the FR targeting may be compromised since absorption of these apoproteins on the surface of DiR-LDL-FA could develop an increased affinity for scavenger receptors and LDLR. This would, in turn, direct DiR-LDL-FA for hepatic uptake and degradation.

In spite of the many factors and processes that compete for the uptake of DiR-LDL-FA, the fraction of particles that were able to reach the tumors were able to effectively target the FR. All in all these results indicate that the rerouting strategy for targeting LDL to alternate receptors for in vivo cancer applications is a viable and promising approach. Future studies will optimize the degree of ligand conjugation for this LDL rerouting strategy. Alternatively, different sizes of rerouted lipoproteins will be utilized for various cancer applications (for example, using the larger VLDL for targeting tumor neovasculature and using the ultra small HDL for targeting solid tumors because of the ease with which it transverses the vascular endothelium[8]).

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Supporting Information Available: Procedures for the synthesis and evaluation of DiR-LDL-FA, biodistribution of DiR-LDL-FA with or without FA competition in tissues and tumor extracts at 24 h postinjection, and comparative real time in vivo fluorescence imaging of mice with or without FA inhibition. This material is available free of charge via the Internet at http://pubs.acs.org.

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