Mechanistic Insights into LDL Nanoparticle-Mediated siRNA Delivery
Honglin Jin,†‡§ Jonathan F. Lovell,†‡∥ Juan Chen,‡ Qiaoya Lin,†‡§ Lili Ding,‡ Kenneth K. Ng,‡∥ Rajendra K. Pandey,⊥ Muthiah Manoharan,⊥ Zhihong Zhang*,† and Gang Zheng*,†‡§∥
†Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics-Huazhong University of Science and Technology, Wuhan, China
‡Ontario Cancer Institute and Campbell Family Cancer Research Institute, University Health Network, Toronto, Canada
§Department of Medical Biophysics and ∥Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Canada
⊥Alnylam Pharmaceuticals, Boston, Massachusetts, United States
*Supporting Information

ABSTRACT: Although small interfering RNA (siRNA) can silence the expression of disease-related genes, delivery of these highly charged molecules is challenging. Delivery approaches for siRNAs are actively being pursued, and improved strategies are required for nontoxic and efficient delivery for gene knockdown. Low density lipoprotein (LDL) is a natural and endogenous nanoparticle that has a rich history as a delivery vehicle. Here, we examine purified LDL nanoparticles as carriers for siRNAs. When siRNA was covalently conjugated to cholesterol, over 25 chol-siRNA could be incorporated onto each LDL without changing nanoparticle morphology. The resulting LDL-chol-siRNA nanoparticles were selectively taken up into cells via LDL receptor mediated endocytosis, resulting in enhanced gene silencing compared to free chol-siRNA (38% gene knock down versus 0% knock down at 100 nM). However, silencing efficiency was limited by the receptor-mediated entrapment of the LDL-chol-siRNA nanoparticles in endolysosomes. Photochemical internalization demonstrated that endolysosome disruption strategies significantly enhance LDL-mediated gene silencing (78% at 100 nM).

INTRODUCTION
RNA interference (RNAi) is one of the most powerful tools for specific gene suppression and thus holds great potential for therapeutic applications, including treatment of dominant genetic disorders, autoimmune diseases, cancer, and viral infections.1–4 Efficient and safe in vivo delivery of small interfering RNA therapeutics (siRNA) to target tissues is the main challenge for their clinical implementation, since highly negatively charged siRNAs cannot readily penetrate through cell membranes. Because virus-mediated nucleic acid delivery systems are primarily focused on a variety of nonvirus delivery strategies including cationic lipids,5–8 cationic liposomes,9 cationic polymers,10–12 peptide conjugates,13,14 antibody conjugated polyethylenimine,15 gold nanoparticles,16,17 quantum dots,18 and modified siRNAs.19 Cationic lipids, liposomes, and polymers (such as PEI) form complexes with anionic siRNA through electrostatic interactions can enhance intracellular delivery of siRNAs. However, most cationic delivery systems have toxicity issues,20–22 with the exception of some of the naturally derived systems, such as chitosan, which are nontoxic but are heterogeneous with large size variation.23 The internalization mechanism of cell penetration peptides (CPPs) is still unresolved.24 While inorganic nanoparticles, such as quantum dots and gold nanoparticles have shown siRNA delivery potential, researchers are still optimizing surface chemistries and studying their long-term toxicity.25,26

LDL nanoparticles are endogeneous nanocarriers possessing many advantages for drug delivery: a homogeneous size (19–25 nm) below 40 nm, biocompatibility and biodegradability,
intrinsic targeting with customizable alternative targeting. All these key features make LDL an attractive nanocarrier for siRNA delivery. Cholesterol modified siRNA (chol-siRNA) has been developed for systemic administration using a noncationic delivery system, resulting in in vivo gene silencing at a dose of 50 mg/kg. The mechanism of delivery occurred through specific interactions between chol-siRNA and serum lipoproteins, and the resulting complexes with targeted cells. Further, when chol-siRNA was preloaded onto murine HDL (1:1 ratio), significant enhancement of gene silencing efficiency was observed. Indeed, we have recently examined HDL mimetics as candidates for siRNA delivery which use a separate internalization pathway. Alternatively, the versatile apolipoprotein LDL has previously been used for the delivery of cholesterol-modified antisense oligonucleotides. Among these methods, photochemical internalization (PCI), which allows the light-induced release of endocytosed macromolecules into the cytoplasm, was chosen. The mechanism involves breakdown of endosomal/lysosomal membranes by amphiphatic photo-sensitive agents, such as disulfonated aluminum phthalocyanine (AlPcS2a) or meso-tetraphenylporphine disulfonate (TPPS2a). This powerful technology has been investigated further as LDL-chol-siRNA could be achieved using LDL nanoparticles. It is known that cellular uptake of LDL nanoparticles is mediated by the LDL receptor and this pathway often leads to the entrapment of LDL and its loading drug in lysosomes and this problem might be overcome by using light-triggered endosomal disruption techniques. Among these methods, photochemical internalization (PCI), which allows the light-induced release of endocytosed macromolecules into the cytoplasm, was chosen. The mechanism involves breakdown of endosomal/lysosomal membranes by amphiphatic photosensitizers, such as disulfonated aluminum phthalocyanine (AlPcS2a) or meso-tetraphenylporphine disulfonate (TPPS2a). This powerful technology has been investigated for the delivery of a variety of drugs, including siRNAs. Recently, we demonstrated that cytosolic release of LDL loaded cargo using PCI was feasible and the efficiency was dependent on the cargo loading method. Direct endolysosomal disruption by PCI resulted in efficient intracellular cytosolic release of surface-loaded cargos and partial release of protein-loaded cargo. Thus, in theory, PCI is well-suited to improve LDL-mediated siRNA delivery efficiency.

### EXPERIMENTAL PROCEDURES

**Materials.** LDL was isolated by sequential ultracentrifugation of human plasma using previously described methods, and was further purified by AKTA fast protein liquid chromatography (FPLC) system (Amersham Biosciences) equipped with a HiLoad 16/70 Superdex 200 pg column. Human plasma was obtained from the local blood transfusion services and usage was approved by the University Health Network’s research ethics board. The PCI agent AlPcS2a was purchased from Frontier Scientific company. All siRNAs were obtained using a Nanosizer (Zetasizer Nano-ZS90; Malvern Instruments, Malvern, UK).

**Conical Microscopy.** HepG2 (Human hepatocellular liver carcinoma cell line) and ldlA7 (LDL receptor-deficient Chinese hamster ovary cells) cells were seeded in 8-well cover glass bottom chambers (Nunc Lab-Tek, Sigma-Aldrich) (3 × 10⁴/well) for confocal microscopy imaging. Briefly, cells were incubated with cell culture medium containing 10% FBS and LDL-chol-si-Cy5.5 or (Fluo-BOA)LDL-chol-si-Cy5.5 at a chol-siRNA concentration of 400 nM for 3 h at 37 °C. For the inhibition study, a 20-fold excess of LDL was added. Confocal imaging was performed using a Hitachi H-7000 transmission electron microscope (TEM) equipped with a digital image acquisition system to determine the morphology of an aqueous dispersion of LDL and LDL-chol-siRNA nanoparticles with 0.5% uranyl acetate as the negative stain. The volume size distribution and zeta potential of LDL and LDL-chol-siRNA were obtained using a Nanosizer (Zetasizer Nano-ZS90; Malvern Instruments, Malvern, UK).

**Evaluation of siRNA Loading on LDL.** LDL was mixed with control chol-siRNA at various molar ratios in PBS. After 30 min incubation, the mixture was assayed for electrophoretic mobility on a 0.8% agarose gel. The siRNA signal was visualized by UV transillumination with GelRed stain and the LDL protein signal was visualized by Coomassie blue stain.

**Preparation of LDL-chol-si-apoB Nanoparticles.** LDL was incubated with chol-si-apoB (4.29 nmol) at 15:1, 25:1, 30:1, 50:1 molar ratio of chol-si-apoB:LDL at room temperature for 30 min in 1 mL PBS (0.1 M NaCl, pH 7.5). The chol-si-apoB/LDL was then purified by FPLC equipped with a HiLoad 16/70 Superdex 200 pg column and eluted with Tris-buffered saline (10 mM Tris-HCl, containing 0.15 M NaCl, 1 mM EDTA, pH 7.5) at a flow rate of 1 mL/min. The chol-siRNA/LDL complex with a molar ratio of 25:1 had no free chol-si-apoB peak and had maximum siRNA loading on LDL. Thus, it was selected as the optimal formulation, and the resulting LDL-chol-si-apoB nanoparticles were further filtered through a 0.2 μm filter (Millipore) and stored at 4 °C for in vitro studies.

**Preparation of LDL-chol-si-Cy5.5 and (Fluo-BOA)LDL-chol-si-Cy5.5.** LDL-chol-si-Cy5.5 was prepared in the same way as LDL-chol-si-apoB by replacing chol-si-apoB with chol-si-Cy5.5. LDL-chol-si-Cy5.5 was prepared similarly by replacing LDL with (Fluo-BOA)LDL and replacing chol-si-apoB with chol-si-Cy5.5. LDL-chol-si-Cy5.5 was prepared by a previously described procedure. The resulting nanoparticles were filtered through a 0.2 μm filter and stored at 4 °C for imaging studies.

**Measurement of LDL Protein Concentration.** The LDL protein concentration in the LDL-chol-siRNA particles was measured using the modified Lowry protein assay (Sigma-Aldrich, St. Louis, MO).

**Morphology, Size, and Surface Charge Measurement.** Transmission electron microscopy (TEM) was performed using a Hitachi H-7000 transmission electron microscope (Hitachi, Inc., Japan) equipped with a digital image acquisition system to determine the morphology of an aqueous dispersion of LDL and LDL-chol-siRNA nanoparticles with 0.5% uranyl acetate as the negative stain. The volume size distribution and zeta potential of LDL and LDL-chol-siRNA were obtained using a Nanosizer (Zetasizer Nano-ZS90; Malvern Instruments, Malvern, UK).
**Cell Toxicity Study.** HepG2 cells were seeded in a 96-well (1 × 10^4/well) plate and cultured in DMEM medium containing 10% FBS and antibiotics (denoted as complete medium) for 2 days. Experiments were started, after one quick wash with PBS buffer, by addition of 200 μL of complete medium containing various concentration of chol-si-apoB or LDL-chol-si-apoB. After 48 h incubation at 37 °C with 5% CO₂, the cells were washed twice with PBS buffer and incubated with 150 μL of complete medium with MTT tracer, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Invitrogen) at 0.5 mg/mL for 1 h. The medium was then replaced with 70% isopropanol in 0.1 M HCl solution, the plate was shaken for 20 min, and absorbance was measured at 570 nm to determine the viability relative to the untreated control.

**RNAi Treatment.** To investigate the apoB gene inhibition, HepG2 cells were first seeded in a 6-well (2 × 10^5/well) plate in complete medium and incubated for 2 days. The medium was then replaced with 500 μL of fresh complete medium containing various concentrations of chol-si-apoB or LDL-chol-si-apoB for 48 h incubation. The cells were then harvested to check apoB mRNA expression by the real-time reverse transcription polymerase chain reaction (RT-qPCR).

**PCI Treatment.** HepG2 cells were seeded in a 6-well (2 × 10^5/well) plate and cultured in complete medium for 30 h. After incubation with 10 μg/mL of AlPcS₂₅₀ in complete medium for 18 h, cells were then washed with PBS and treated with LDL-chol-si-apoB in complete medium for 3 h. The light irradiation was performed with a 660 nm light box under various treatment durations. The cells were allowed to continue growth to total incubation time of 48 h (from the time of siRNA treatment) and harvested for further analysis.

**RESULTS**

To investigate the loading of siRNAs onto LDL, a control siRNA sequence was initially used. The chol-siRNA or unmodified siRNA (siRNAs lacking of cholesterol moiety) was incubated with LDL at molar ratios ranging from 0:1 to 80:1 for 30 min. Agarose gel electrophoresis of chol-siRNA/LDL showed comigration of siRNA and LDL, indicating that...
chol-siRNA could be readily incorporated onto LDL (Figure 1A). Without cholesterol modification, siRNA did not associate with LDL (Figure 1B). Incorporation of chol-siRNA increased the particle size by up to 10 nm from 0:1 to 30:1 initial chol-siRNA:LDL ratio. Further increase of the initial chol-siRNA to LDL ratio had no impact on the nanoparticle size. An inverse correlation between zeta potential and chol-siRNA loading was also observed (Figure 1C), demonstrating the negatively charged chol-siRNA bound to LDL. No stable binding between LDL and unmodified siRNA was observed by zeta potential. These data suggest that chol-siRNA could be stably loaded on LDL with a high payload (30 of chol-siRNA per particle) and demonstrate the critical role of the cholesterol moiety for LDL association. LDL and chol-siRNA fully associated within minutes and the resultant negatively charged particles showed more migration in a gel when compared with LDL (Figure 1A). TEM images (Figure 1D) confirmed that incorporation of chol-siRNA on LDL did not change the morphology or homogeneous size distribution of the nanoparticles.

We next examined LDL-chol-siRNA that targeted the apoB gene (LDL-chol-si- apoB). As shown in Figure 2A, FPLC can separate LDL-chol-si-apoB (retention time <58 min), LDL (67 min) and unloaded free siRNAs (102 min) (Figure 2A). At the initial chol-siRNA/LDL complex ratio of 30:1 (chol-si-apoB: LDL), a free siRNA peak was present in the FPLC profiles. When adjusting the complex ratio to 25:1, negligible unloaded free chol-siRNA was present (SI Figure 1S), suggesting that the initial chol-siRNA/LDL complex ratio of 25:1 gave an optimal formulation for LDL-chol-si-apoB preparation. This optimized formulation was used for further functional and imaging studies. LDL-chol-si-apoB nanoparticles appeared monodisperse by TEM (Figure 2B) and possessed a hydrodynamic diameter of 31 nm based on dynamic light scattering, while the unmodified LDL nanoparticles had a hydrodynamic diameter of 23 nm (Figure 2C). Loading chol-si-apoB onto LDL nanoparticles dramatically changed the surface charge of the nanoparticle evidenced by the zeta potential shift from −5.3 mV of LDL to −23.9 mV of LDL-chol-si-apoB.

We next examined the specific uptake of the LDL-chol-si-apoB nanoparticles using the Cy5.5-labeled chol-si-apoB. The LDL-chol-si-Cy5.5 nanoparticles were incubated with two cell lines: HepG2 cells that have high expression of the LDL receptors and LdA7 cells that have low expression. Confocal microscopy demonstrated that, after 3 h incubation, significant uptake of chol-si-Cy5.5 occurred within the HepG2 cells (LDLR⁺), whereas LdA7 (LDLR⁻) cells displayed 10-fold less uptake (Figure 3A,B). Moreover, the delivery of chol-si-Cy5.5 was inhibited by adding a 20-fold excess of unlabeled LDL as a competitive inhibitor during the incubation. These results show that targeted siRNA delivery could be achieved using LDL.

To investigate the RNAi efficiency of LDL-chol-si-apoB, HepG2 cells were treated with various concentrations of LDL-chol-si-apoB nanoparticles or chol-si-apoB alone in the presence of complete growth medium (with 10% FBS) at 37 °C for 48 h. LDL-chol-si-apoB showed enhanced gene silencing efficiency at all the concentrations when compared with chol-si-apoB alone (Figure 4A). No concomitant cell toxicity was observed when the cells were treated with either LDL-chol-si-apoB nanoparticles or chol-si-apoB alone (Figure 4B). These data suggest that LDL is a safe nanocarrier capable of functional delivery of chol-siRNA. However, increasing LDL-chol-si-apoB concentrations only resulted in moderate decreases in apoB mRNA expression (apoB gene expression was reduced by 38%, 46%, and 60% with LDL-chol-si-apoB concentrations at 100, 300, and 600 nM, respectively). This limited knockdown was probably due to inefficient release of siRNAs into the cytoplasm, which limited the siRNA efficiency.

To elucidate the siRNA delivery mechanism using LDL, the intracellular behavior of the LDL-chol-si-apoB was investigated on HepG2 cells incubated with a dual dye labeled particle, (Fluo-BOA)LDL-chol-si-Cy5.5, where LDL was labeled with Fluo-BOA in the core and siRNA was labeled with Cy5.5. Confocal microscopy was used to investigate whether the chol-siRNA separated from the LDL after cellular internalization. After 3 h incubation with (Fluo-BOA)LDL-chol-si-Cy5.5, significant colocalization between Fluo-BOA and Cy5.5 signals was observed (Figure 5A), implying the nanoparticle and cargo were intact at that time. Further confocal studies showed that chol-si-Cy5.5 colocalized with transferrin (an endosomal marker, Figure 5B) and lysotracker (a lysosomal marker, Figure 5C) at the 3 h incubation time point, confirming that LDL delivered chol-siRNA into endo/lysosomes organelles of cells via receptor mediated endocytosis.
PCI is a useful technique that allows the light-induced release of endocytosed macromolecules into the cytoplasm. To bypass the endolysosomal trafficking of siRNAs brought in by LDL, we combined PCI with LDL-chol-siRNA to investigate the ability to facilitate the release of chol-siRNA and enhance delivery.

Figure 3. Targeted delivery of siRNAs via the LDL receptors using LDL-chol-siRNA. (A) Cy5.5-labeled fluorescent siRNA (chol-si-Cy5.5) was used to track the intracellular uptake of siRNAs. Confocal imaging was performed on HepG2 cells (LDLR+) and ldlA7 (LDLR−) cells treated with 400 nM of LDL-chol-si-Cy5.5 for 3 h. Competition uptake study was carried out by adding 20-fold excess of LDL. The nuclei were stained with Hoechst 33258 and are shown in blue staining and the internalized chol-si-Cy5.5 appears red. The scale bar is 20 μm. (B) Measurement of the average fluorescence intensity of cells from confocal images. Data was acquired from at least 20 cells from two fields of view (**p < 0.001).

Figure 4. Evaluation of (A) gene knock-down and (B) cytotoxicity of LDL-chol-si-apoB and chol-si-apoB to HepG2 cells at various concentrations (n = 3, * p < 0.05). LDL alone and PBS buffer were used as controls. The cell visibility was determined by MTT assay (n = 3).

Figure 5. Investigation of cellular delivery pathway of LDL-chol-siRNA. (A) HepG2 cells were treated with (Fluo-BOA)LDL-chol-si-Cy5.5, where LDL was labeled with Fluo-BOA and siRNA was labeled with Cy5.5. The signals of Fluo-BOA (Green) and Cy5.5 (Red) colocalized at 3 h incubation. The nuclei were stained with Hoechst 33258 (blue) and the scale bar is 30 μm. (B) chol-si-Cy5.5 was found colocalized with transferrin, an early endosome marker, after 3 h incubation with HepG2 cells. The scale bar is 10 μm. (D) chol-si-Cy5.5 was also colocalized with lystrotacker after 3 h incubation with HepG2 cells. The scale bar is 10 μm.
RNAi efficiency. To use spectrally distinct wavelengths with the PCI agent AlPcS2a (excitation, 675 nm; emission, 702 nm), FITC (excitation, 495 nm; emission, 525 nm) labeled chol-siRNA (chol-si-FITC) was used for the imaging of PCI release study. Although LDL delivered chol-siRNA into endo/lysosomes of cells (Figures 3 and 5), a very weak chol-si-FITC signal was observed in endo/lysosomes of cells (SI Figure S2) probably due to the fluorescence quenching of FITC at low pH condition (endo/lysosomes), which is consistent with the observation of Carraway et al.43 Following light irradiation with PCI agent AlPcS2a, strong signal of chol-si-FITC (green channel) escaped from endo/lysosome and spread to the cytoplasm of the cells in 5 min, demonstrating cytosolic release of siRNAs from endo/lysosomes (SI Figure S2). To examine gene knockdown, PCI light irradiation was performed using a 660 nm light box with a power density of 2.4 mW/cm². Since photosensitizer-induced cytotoxicity is an inherent issue with PCI, we first optimized the treatment condition by adjusting the light irradiation time. It was found that no remarkable cell death occurred after HepG2 cells incubated with AlPcS2a (PCI agent) for 18 h and received light irradiation up to 180 s duration (SI Figure S3). Thus, the PCI conditions of 18 h for PCI agent incubation, and less than 180 s for light irradiation were selected for gene knockdown study. Followed with the preincubation of AlPcS2a, 3 h treatment of 200 nM LDL-chol-si-apoB, and light irradiation for 60 s, HepG2 cells were then harvested at 48 h after RNAi treatment. RT-qPCR analysis with normalization to a housekeeper gene (Figure 6A) demonstrated enhancement of apoB gene silencing by PCI, with the mRNA expression reducing from 46.3% to 32.9% (p < 0.05), while a mock siRNA with LDL (LDL-chol-si-mock) did not show obvious gene knockdown either with or without PCI. These results suggest that the specific gene silencing was induced by the specific sequence of siRNA and PCI is a potential option to enhance the gene silencing efficacy in the context of using LDL as a delivery vehicle. To further investigate the PCI efficacy, cells incubated with a lower concentration (100 nM) of LDL-chol-si-apoB were treated by light for 0, 30, 60, or 180 s, respectively. A clear PCI induced gene silencing was observed with the mRNA expression reducing from 62% (no light treatment) to 53%, 39%, and 22% upon light treatment of 30, 60, and 180 s, respectively. Under the longest irradiation time of 180 s, RNAi efficiency was significantly increased by 2.1-fold (p < 0.01). In addition, cells that were exposed to PCI but not LDL-chol-si-apoB did not show any apoB gene silencing (Figure 6B, right column). These data indicate that improved RNAi efficiency was achieved as a result of the cytosolic release of chol-siRNA through PCI.

**DISCUSSION**

In this study, LDL nanoparticles were used as nanocarriers for chol-siRNA. Each LDL nanoparticle could stably incorporate at least 25 molecules of chol-siRNA while maintaining structural stability. The resultant nanoparticles had a mean size of ∼30 nm, which is suitable to navigate through the restricted interfibrillar openings (<40 nm) commonly present in solid tumors,44 making them powerful carriers for the delivery of intracellular active siRNAs via systemic administration. Targeted delivery of chol-siRNA can be achieved using LDL nanoparticles because of high LDL receptor expression in a variety of cancer cells, which is believed to provide cancer cells with the necessary lipid substrates required for membrane synthesis. Although this delivery platform had superior knockdown efficiency to chol-siRNA alone, use of the LDL receptor resulted in receptor mediated endocytosis and entrapment of chol-siRNA in endolysosomal compartments. The lack of a specific cytosolic release mechanism probably limited knockdown efficiency. This was confirmed by improved knockdowns when a PCI approach was used, which released siRNAs to cytoplasm. Thus, LDL nanoparticles can be used for the cellular delivery of chol-siRNA, and PCI provides efficient...
endolysosomal membrane disruption and spatiotemporal delivery of cytosolic release. The chol-siRNA delivery system in the study for targeting apoB gene was used as a model proof of principle. However, the apoB gene might not be an ideal target for LDL receptor mediated delivery, since both LDL and apoB protein participate in the process of cholesterol metabolism, that may induce unexpected effects in therapeutic applications. The targeted siRNAs could be further adapted to other genes and diseases to engage in other potent mechanisms, such as the genes for induction of cancer cell apoptosis. Another challenge of using LDLR as a target for cancer theranostics is that some normal cells, such as liver hepatocytes, also express LDL receptors. PCI provides a second layer of control in the LDL delivery system for highly selective drug release. Moreover, as we reported previously, the LDL delivery system is not limited to the LDL receptor. For situations where the LDL-R is not highly expressed, a method for targeting LDL nanoparticles to other cancer receptors is warranted. Thus, the combination of LDL nanocarriers and PCI approach provide a useful strategy and exquisite control for therapeutic use of siRNAs. In addition, there are at least three ways for incorporating diagnostic and therapeutic agents into LDL, covalent attachment to amino acid residues of apoB-100 protein (protein modification), intercalation into the phospholipids monolayer (surface loading) and reconstitution into the lipid core of LDL (core loading). Since the interaction between LDL and chol-siRNA is based on surface loading, the core lipid of LDL still can be replaced with other drugs for multimodality treatment or image-guided therapy, and this LDL system can be extended to other lipoprotein-based nanoparticles or lipoprotein-mimetic peptide-based delivery systems.

**ASSOCIATED CONTENT**

Supporting Information

FPLC purification of LDL-chol-si-apoB complexes, imaging of PCI directed cytosolic release of chol-siFITC after LDL mediated endosytosis and cytotoxicity of LDL-chol-siRNA in different experimental conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

**Corresponding Author**

*Dr Gang Zheng, Ontario Cancer Institute, TMDT 5-363, 101 College Street, Toronto, ON, MSG 1L7, Canada, E-mail: gang.zheng@uhnres.utoronto.ca. Dr Zhihong Zhang, Britton Chance Center for Biomedical Photonics, Huazhong University of Science and Technology, Wuhan 430074, China. E-mail: cyyzh@mail.hust.edu.cn.

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