As a ligand, aptamers possess several advantages over other ligands that are used in drug delivery such as antibodies. First, the production of aptamers doesn't require any biological system and, hence, is much easier to scale up with low batch-to-batch variability [11]. Second, aptamers are quite thermally stable and can be denatured and renatured multiple times without any loss of activity. Third, aptamers can be chemically modified to enhance their stability in biological fluids, because of their smaller size; they are able to easily and rapidly diffuse into tissues and organs and thus permit faster targeting in drug delivery. Lastly, conjugation chemistry for attaching various imaging labels or functional groups to aptamers is orthogonal to nucleic acid chemistry, and hence they can be readily introduced during aptamer synthesis. Extensive research on aptamers indicate that they have great potential for use in a variety of areas, including diagnosis, therapy, biomarker identification, and, most promising, as a targeting ligand for developing new drug delivery systems [8–12]. Macugen (Pegaptanib) is the first nucleic acid aptamer that was approved by the US Food and Drug Administration in December 2004 as an anti-angiogenic therapeutic agent for neovascular (wet) age related macular degeneration. A variety of aptamers against other molecular targets are currently undergoing clinical investigation [13,14].

As of this writing, liposomes are the most successful drug delivery system available. From the first discovery to date, many liposomal formulations have been approved by the US Food and Drug Administration, and many are in preclinical and clinical trials in different fields [15–18]. This class of nano-particles improve the solubility, toxicity profile, and unfavorable pharmacokinetics of a chemotherapeutic. However, therapeutic efficacy remains a big challenge and is largely unchanged. As a result, the development of a tool to allow constant and selective delivery would be highly desirable. The key problems of drug therapy such as biodistribution throughout the body and targeting to specific receptors could be improved by using a ligand based liposomal formulation [19]. PEGylated liposomes, also known as stealth liposome posses some advantages, including a longer circulation time, and have the ability to passively accumulate in tumor tissues or organs, although, they have been reported to have insufficient cellular-uptake and endosomal escape properties, a fact that reduces the pharmacological effect of the drug, this phenomenon is commonly referred to as the PEG-dilemma. To increase the efficacy of delivery to target tissues, aptamer modified liposomes can be considered as good candidates. After Willis's pioneering work on 1998 [20], the drug delivery using aptamer modified liposomes have been investigated well [21–23]. A few *in vivo* research studies were also initiated and the aptamer mediated liposomal active targeting strategy appears to hold considerable promise for use as a liposomal drug delivery system [24,25]. Many of the aptamer-liposome drug delivery systems have been applied to targeting cancer parenchymal cells and not the tumor vasculature. The goal of this study was to examine the use of target-specific ligand aptamer modified liposomes, an alternative promising approach, to reduce the side effects associated with PEG and thus, allow targeting to the tumor vasculature with better efficacy [26].

Angiogenesis-dependant tumor growth was first reported by Folkman in 1971 [27]. Preventing or inhibiting angiogenesis is associated with the increased vascularity necessary for tumor progression and metastasis. Metastases are the cause of 90% of all human cancer deaths. Chemotherapy of cancer metastases, which are effective in some patients, are often associated with significant toxicity, due to the nonspecific distribution of cytotoxic drugs which limits the maximum allowable dose [28,29]. Tumor blood vessels provide nutrients and oxygen, and remove waste from tumor tissue, thus enhancing tumor progression. Tumor blood vessels have been shown to differ from their normal counterparts in that they show leakiness and have a basement membrane that is thick and uneven. This suggests that tumor endothelial cells may well express surface markers that are different from those found in normal cells [30,31]. Our rationale for targeting tumor endothelial cells in our current project is based on the following assumptions, Tumor endothelial cells can support many tumor cells, and thus, targeting endothelial cells might be a much more effective strategy than targeting actual tumor cells themselves. In fact, active targeting can be achieved by the efficient recognition of specific antigens that are expressed on the cell surface proteins of tumor cells but are not expressed on normal cells [32–36]. Therefore, the ligand attached on the surface of PEGylated liposomes such as Apt-PEG-LPs can be enhanced the cellular uptake.

We recently isolated a DNA aptamer AraHH001 (Kd = 43 nM) that is selectively expressed on the mates of different origin and does not bind to healthy endothelial cells. Additionally, this aptamer has a high internalization capacity [37], providing a means for the intracellular delivery of drugs or gene therapy that are themselves not permeable to cells. For this reason, we selected this high affinity aptamer for use in the current study. We established a aptamer-based mTECs targeted liposomal drug delivery system which enhanced the uptake into target mTECs compared to PEGylated liposomes, and conducted a detailed study of the uptake mechanism and intracellular trafficking for this system.

2. Methods

2.1. Synthesis of a DNA aptamer AraHH001 conjugated maleimide-PEG_{2000}-DSPE

The conjugation of the AraHH001 aptamer (ACGTACCGACTTCGTATGCCAACAGCCCTTTATCCACCTC) (100 nmol) reacted with a 5 times excess of maleimide-PEG_{2000}-DSPE was performed by a gentle overnight soaking in a Bio-shaker at room temperature. AraHH001 was purchased from Sigma-Genosys. For the conjugation reaction, the disulphide (S–S) bonds of AraHH001 were first cleaved by treatment with an excess TCEP solution on ice for 30–40 min. After the conjugation reaction, the excess maleimide-PEG_{2000}-DSPE was removed by dialysis (MWCO 3500–5000) in 1% SDS, 50 mM phosphate buffer at pH 7 with the solvent being changed three times at 4 h intervals. Further dialysis was performed in 50 mM ammonium hydrogen carbonate buffer at pH 8.0 by changing the solvent three times at every 4 h interval. The purified aptamer-lipid conjugation was ion-exchanged with Zip-Tip C18 and examined by agarose-gel electrophoresis and MALDI-TOF mass spectroscopy.

2.2. Preparation of liposomes

Liposomes (LPs) formulations were prepared by the standard lipid hydration method. The molar ratio of EPC, Chol and Rhodamine-DOPE was 70:30:1. About 5 mol% of PEG_{2000}-DSPE or Apt-PEG_{2000}-DSPE of the total lipid was added to the lipid solutions during the preparation of the PEG-LPs or Apt-PEG-LPs respectively. All lipids were dissolved in chloroform/ethanol solutions, and, a lipid film was prepared by evaporating all of the solvents under a stream of nitrogen gas. The dried lipid film was hydrated by adding HEPES buffer (10 mM, pH = 7.4) for 10 min at room temperature, followed to the sonication for approximately 30 s–1 min in a bath type sonicator (AU-25 C, Aiwa, Tokyo, Japan). The average size and diameter of liposomes were measured by using a Zetasizer Nano ZS ZEN3600 (Malvern Instrument, Worcestershire, UK).

2.3. Isolation of mouse tumor endothelial cells (mTECs)

All experiments involving animals and their care were carried out consistent with Hokkaido University guidelines, and protocols approved by the Institutional Animal Care and Use Committee. Endothelial cells were isolated as previously described [32–36]. Briefly, normal endothelial cells NECs were isolated from the dermis as controls. TECs were isolated by magnetic bead cell sorting using an IMag cell separating system (BD Bioscience). CD31-Positive cells were sorted and plated on 1.5% gelatin-coated culture plates and grown in EGM-2 MV (Clonetics, Walkers, MD) and 15% FBS. Diphtheria toxin (DT) (500 ng/mL, Calbiochem, San Diego, CA) was added to the TEC subcultures to kill any remaining human tumor cells. Human cells express heparin-binding EGF-like growth factor (hHB-EGF), a DT-receptor. However, DT does not interact with mouse HB-EGF and murine ECs survive this treatment.

2.4. Maintenance of cell cultures

Human renal cell carcinoma, OS-RC-2 cells were culture in RPMI-1640, containing 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). Primary cultured TECs were cultured using a special medium, namely EGM-2 MV (Lonza). To prevent microbial growth, penicillin (100 unit/mL) and (100 μg/mL)

streptomycin were added to the EGM-2 MV. Cell cultures were maintained at 37 °C in a 5% CO_2 incubator at 95% humidity. For regular cell cultures a 0.1% trypsin solution was used to dissociate the cells from the surface of the culture dish. However, during the entire selection of a DNA aptamer, flow cytometry assay and during aptamer targeted protein purification, RepCell was used (cell seed Inc., Tokyo, Japan).

2.5. Quantitative cellular uptake analysis of Apt-PEG-LPs in mTECs by spectrofluorometer

To perform a quantitative cellular uptake analysis, 4×10^4 cells were seeded per cm^2 in 24-well plates (Corning Incorporated, Corning, NY, USA) and incubated overnight at 37 °C in an atmosphere of 5% CO_2, and in 95% humidity. On the next experimental day, medium from cells in 24 well plates was removed by aspiration and the cells then washed with warm PBS once. A different rhodamine labeled liposomal solution was then added to the cells, followed by incubation for 3 h at 37 °C in an atmosphere of 5% CO_2, and in 95% humidity. After 3 h of incubation, the cells were washed with 1× warm PBS supplemented with 100 nM cholic acid twice and the cells were then incubated with 1× Reporter lysis buffer at −80 °C to lysis and after 20 min, they were put on ice to melt treated cell suspensions were treated with the different liposome solution in 24-well plates. Finally, the lysed solution was centrifuged at 12,000 rpm, for 5 min at 4 °C to remove cell debris. The efficiency of cellular uptake in terms of the Fluorescence intensity of Rhodamine in the supernatant solution was measured using a FP-750 Spectrofluorometer (JASCO, Tokyo, Japan) at the excitation and emission range (550–590 nm).

2.6. Qualitative cellular uptake analysis Apt-PEG-LPs in mTECs by confocal laser scanning microscopy (CLSM)

For performing Confocal microscopy, 2×10^5 mTECs were seeded per 35-mm glass bottom dish (Iwaki, Chiba, Japan) in 2 mL of culture medium 24 h before the experiment in a 37 °C incubator under an atmosphere of 5% CO_2, and in 95% humidity. On the next experimental day, the medium was removed from the cells by aspiration and the cells were then washed once with 2 mL of 1× PBS, and then incubated with 5 mol% of the total lipid of PEG-LPs, or Apt-PEG-LPs in Kreb's buffer for 3 h at 37 °C. After 2.5 h of incubation, 20 μl of Hoechst 33342 (1 mg/mL) was added to stain the nuclei and the suspension was re-incubated for an additional 30 min. The medium was then removed and the cells were washed twice with a 1 mL of 1× PBS supplemented with cholic acid (10 nM). Finally, 1 mL of Krebs buffer was added and the cells were analyzed under confocal microscopy (A1 Confocal Laser Microscope System, Olympus Instruments Inc., Tokyo, Japan).

2.7. Intracellular trafficking of Apt-PEG-LPs in mTECs via confocal laser scanning microscopy (CLSM)

mTECs were seeded in 35 mm glass bottom dish with 2 mL of medium and incubated for 24 h. The cell density was 2×10^5 cells/glass bottom dish. On the next experimental day, the cells were incubated with 5 mol% of the total lipid of Apt-PEG-LPs and PEG-LPs in Krebs buffer for 3 h at 37 °C under an atmosphere with 5% CO_2 and in 95% humidity. The cells were stained with LysoTracker green (DND-26) 1 (μg/mL) for 30 min at 37 °C. After 2–3 washings with 1× PBS supplemented with 10 nM cholic acid, the cells were examined by confocal laser scanning microscopy, as described above.

2.8. Effect of uptake in competition of labeled Apt-PEG-LPs with excess unlabeled Apt-PEG-LPs

Initially to confirm the pathway of aptamer modified PEGylated liposomes, a competition uptake assay was performed within labeled and unlabeled Apt-PEG-LPs in 1:2 M ratios in mTECs. A total 2×10^5 mTECs/glass bottom dish was prepared in the same manner as described above. Labeled and unlabeled Apt-PEG-LPs in Krebs buffer were subject to incubate in incubator with 3 h at 37 °C under an atmosphere with 5% CO_2 and in 95% humidity. After 2–3 washings with 1 × PBS supplemented with 10 nM cholic acid, the cells were examined by confocal laser scanning microscopy, as described above.

2.9. Investigation of the cellular uptake mechanism using excess unlabeled Apt-PEG-LPs by confocal laser scanning microscope (CLSM)

For the investigation of uptake mechanism, mTECs were prepared as described above. The cells were incubated with labeled and labeled-unlabeled (1:2 ratio) Apt-PEG-LPs (5 mol% of the total lipid) in Kreb's buffer for 3 h at 37 °C under an atmosphere with 5% CO_2 and in 95% humidity. Apt-PEG-LPs were labeled with 1 μL Rhodamine (1 mM). After 2.5 h of incubation, 20 μl of Hoechst 33342 (1 mg/mL) was added to stain the nuclei and the suspension was re-incubated for an additional 30 min. After two to three washings, the cells in Krebs buffer were immediately subjected to analysis by confocal laser scanning microscopy.

2.10. Qualitative and quantitative evaluation of the different receptor mediated endocytic cellular uptake pathway

For the qualitative CLSM studies to investigate the mechanism of internalization of the modified Apt-PEG-LPs, 2×10^5 cells were seeded in a 35 mm glass bottom dish in 2 mL medium and then incubated overnight at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. The cells were washed with 1 mL of 1 × PBS and then pre-incubated with Kreb's buffer for various times in the absence or presence of the following inhibitors: Amiloride (1 mM) for 10 min; Sucrose (0.25 M) for 30 min or Filipin III (1 μg/mL) for 30 min at 37 °C. The various inhibitors were removed by aspiration, followed by washing once with Krebs buffer and the Apt-PEG-LPs were added to the cells, followed by incubation for 1 h at 37 °C. The cells were washed twice by adding 1 mL of PBS supplemented with 100 nM cholic acid. Finally the cells in 1 mL Krebs buffer were observed under the confocal laser scanning microscope.

To quantitatively investigate the mechanism of internalization of the modified Apt-PEG-LPs, 4×10^4 cells were seeded in a 24-well plate (Corning incorporated, Corning, NY, USA) and the plate was incubated overnight at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. The cells were washed with 1 mL of PBS and then pre-incubated with Kreb's buffer for various times in the absence or presence of the following inhibitors: Amiloride (1 mM) for 10 min; Sucrose (0.25 M) for 30 min or Filipin III (1 μg/mL) for 30 min at 37 °C. The various inhibitors were removed by aspiration, followed by washing once with Krebs buffer and then Apt-PEG-LPs were added in the cells to incubate for 1 h at 37 °C. The cells were washed twice by adding 1 mL of PBS supplemented with 100 nM cholic acid. Apt-PEG-LPs were added and the cells were incubated for 1 h at 37 °C. The cells were washed twice by adding 1 mL of PBS supplemented 100 nM cholic acid and the cells lysed with 1× Reporter lysis buffer at −80 °C for 20 min, and, after waiting for more than 20 min on the ice to melt the different liposomes solution, the treated cell suspensions were placed in 24-well plates. Finally the lysed solutions were centrifuged at 12,000 rpm, for 5 min at 4 °C to remove cell debris. The efficiency of cellular uptake in terms of the Fluorescence intensity of Rhodamine in the supernatant solution was measured as described above.

2.11. Qualitative evaluation of the in vivo intratumoral localization of systemically injected Apt-PEG-LPs

Human renal cell carcinoma, 1×10^6 OS-RC-2 cells, were subcutaneously injected on the right flank of mice. When the tumor volume reached 100 mm^3, the tumor-bearing mice were used for *in vivo* evaluation. Regarding the LPs, PEG_{5000}-DSPE was incorporated to circumvent the clearance of LPs via the liver and spleen. Bare LPs were prepared as described above, and PEG_{5000}-DSPE was then post-inserted by incubating the LPs with PEG_{5000} and the Apt-PEG at 60 °C for 30 min (Apt/PEG_{5000}-LPs). For the CLSM study, the fluorescent dye, DiL was administered at 1.0 mol% of the total lipid, and was added to the tubes when lipid film was prepared. Tumor-bearing mice was administered via the tail vein with the LPs at 750 nmol of lipid, and the tumor was then excised under anesthesia 6 h after the injection. To visualize the tumor vessels, FITC-isolectin B4 (Vector Laboratories, Burlingame, CA) was systemically injected into the tumor-bearing mice 10 min before the sample collection. The excised tumor tissue was observed by CLSM (Nikon A1, Nikon Instruments Inc., Tokyo, Japan). The total number of pixel of interest in each confocal image was calculated using the ImagePro-plus software (Media Cybernetics Inc., Bethesda, MD). Co-localization ratio with TECs was calculated according to the following equation; *Co-localization ratio with TECs* = (*yellow pixel*)/(*red pixel* + *yellow pixel*). The above mentioned procedures were approved by the Hokkaido University Animal Care Committee in accordance with the Guidelines for the Care and Use of Laboratory Animals.

3. Results

3.1. Synthesis of DNA aptamer AraHH001 conjugate with maleimide-PEG_{2000}-DSPE

Apt-PEG_{2000}-DSPE was successfully synthesized by the conjugation of a 5-thiol-modified aptamer and 5 equimolar amounts of maleimide-PEG-$DSPE_{2000}$. MALDI-TOF mass was employed to confirm the conjugation (Fig. 1). Excess free lipid was successfully removed by overnight dialysis using 3500–5000 MWCO. The final quantification of Apt-PEG_{2000}-DSPE was done by UV-Visible spectroscopy at 260 nm and the conjugation was ready for preparing liposomes.

3.2. Quantitative cellular uptake analysis of aptamer-modified PEG liposomes on mTECs by spectrofluorometer

To demonstrate the function of our developed aptamer modified PEGylated Nanocarrier system that targeted primary cultured tumor endothelial cells, we first carried out an *in vitro* quantitative cellular uptake experiment using Rhodamine labeled 5 mol% of the total lipid of PEG-LPs and Apt-PEG-LPs on mTECs. The relative fluorescence intensity of Apt-PEG-LPs was found to be almost 3.8

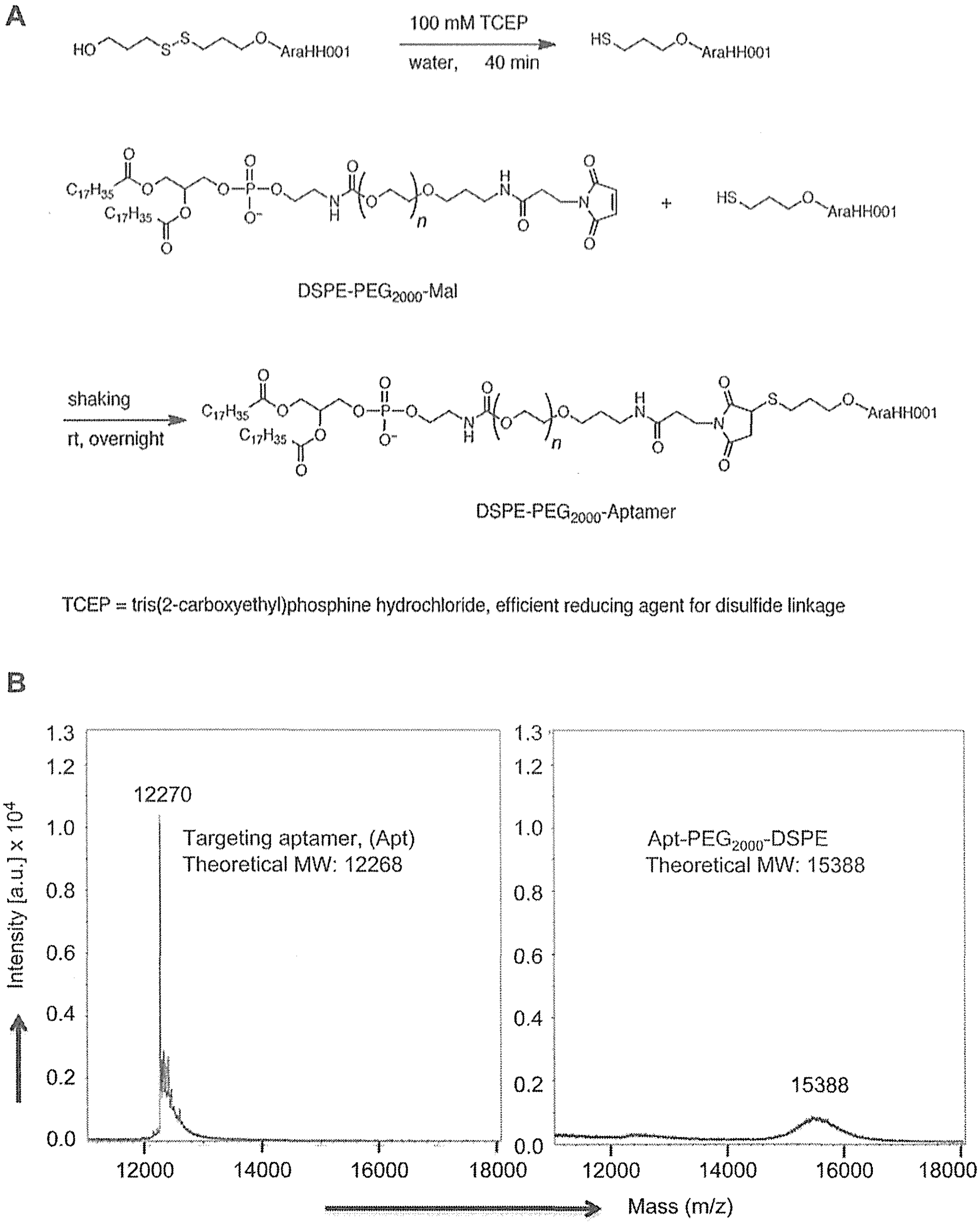

Fig. 1. Conjugation of Apt-PEG_{2000}-DSPE. (A). synthesis of thiol modified aptamer AraHH001 with maleimide-PEG_{2000}-DSPE. Reduced aptamer and excess maleimide-PEG_{2000}-DSPE were reacted in water overnight at 37 °C. (B). MALDI-TOF mass spectrometry was employed to confirm the conjugation.

fold higher than that for PEG-LPs used as the control (Fig. 2). The enhanced cellular uptake in terms of relative fluorescence intensity was statistically significant compared to control PEG-LPs.

3.3. Qualitative cellular uptake study of aptamer-modified PEG liposomes on mTECs by CLSM

The cellular uptake of Apt-PEG-LPs and PEG LPs by mTECs was also tested by CLSM, as shown in (Fig. 3). The cellular uptake of PEG-LP was used as a negative control, showing a very weak fluorescence signal, representing that the only small amount of PEG-LPs were internalized into the cells. Compared to the control, our aptamer AraHH001 modified PEGylated liposomal nano-carrier system resulted in a higher uptake capacity, and at the same time, showed an enhanced ability to recognize the target protein on cell surface receptors.

3.4. Intracellular trafficking of aptamer modified PEGylated liposomes in mTECs by CLSM

To demonstrate the actual localization of internalized aptamer modified PEGylated liposomes and or, either intact or particles that

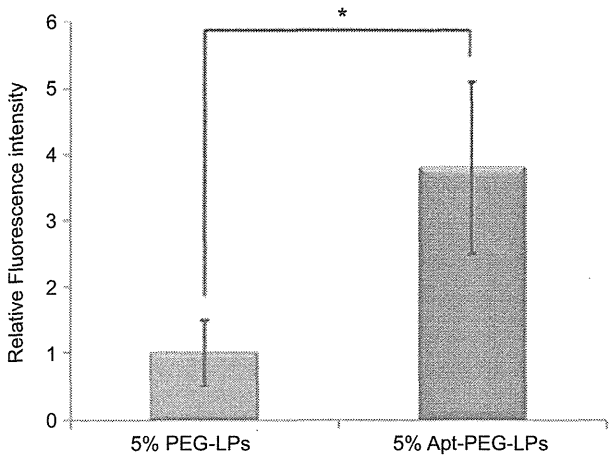

Fig. 2. Quantitative cellular uptake assay of Apt-PEG-LPs. SM-ECs, 4×10^4/24-well were treated with 5 mol% of the total lipid of Apt-PEG-LPs or PEG-LPs for 3 h at 37 °C. The relative cellular uptake is expressed as mean ± SD. Statistical analysis of cellular uptake of Apt-PEG-LPs v's PEG-LPs was performed by unpaired student't test ($n = 5$), $^*P < 0.05$, significant.

had escaped from endosomes and, or, underwent endosomal degradation, Rhodamine labeled Apt-PEG-LPs and PEG-LPs were incubated for 3 h with mTECs. The mTECs were stained with green lysotracker. A CLSM study showed that a certain portion of the Apt-PEG-LPs were merged with lysotracker, indicating that they were located in the lysosomal compartment (Fig. 4A). The remaining portions about 39% appeared as a non-colocalized form into the cells (Fig. 4B). Image Pro software (Media Cybernetics Inc., Bethesda, MD) was applied to count the pixels corresponding to the colocalized and noncolocalized area of Apt-PEG-LPs and PEG-LPs inside the cells.

3.5. Competition with excess unlabeled Apt-PEG-LPs reduced the uptake of Apt-PEG-LPs

To confirm the pathway responsible for the receptor mediated uptake of the Apt-PEG-LPs, we carried out a competition uptake assay with Rhodamine labeled and unlabeled (1:2) Apt-PEG-LPs in mTECs. Only Rhodamine labeled Apt-PEG-LPs was used in this uptake assay as a control (Fig. 5). The competition assay was successful in blocking the target receptor to a certain extent so that uptake inhibition was apparent compared to the control labeled Apt-PEG-LPs.

3.6. Qualitative and quantitative uptake inhibition assay of Apt-mediated liposomes by different endocytic inhibitors

The entry route of cellular uptake of aptamer modified PEGylated liposomes was further examined by the presence of different endocytic pathway inhibitors. Different inhibitors such as Amiloride for macropinocytosis, sucrose for clathrin-mediated, Filipin for caveolae-mediated inhibitors [38] were used to determine the uptake rate for Apt-PEG-LPs. An *in vitro* CLSM uptake study in the presence of different inhibitors showed that the targeted Apt-PEG-LPs were inhibited significantly by clathrin mediated pathways,

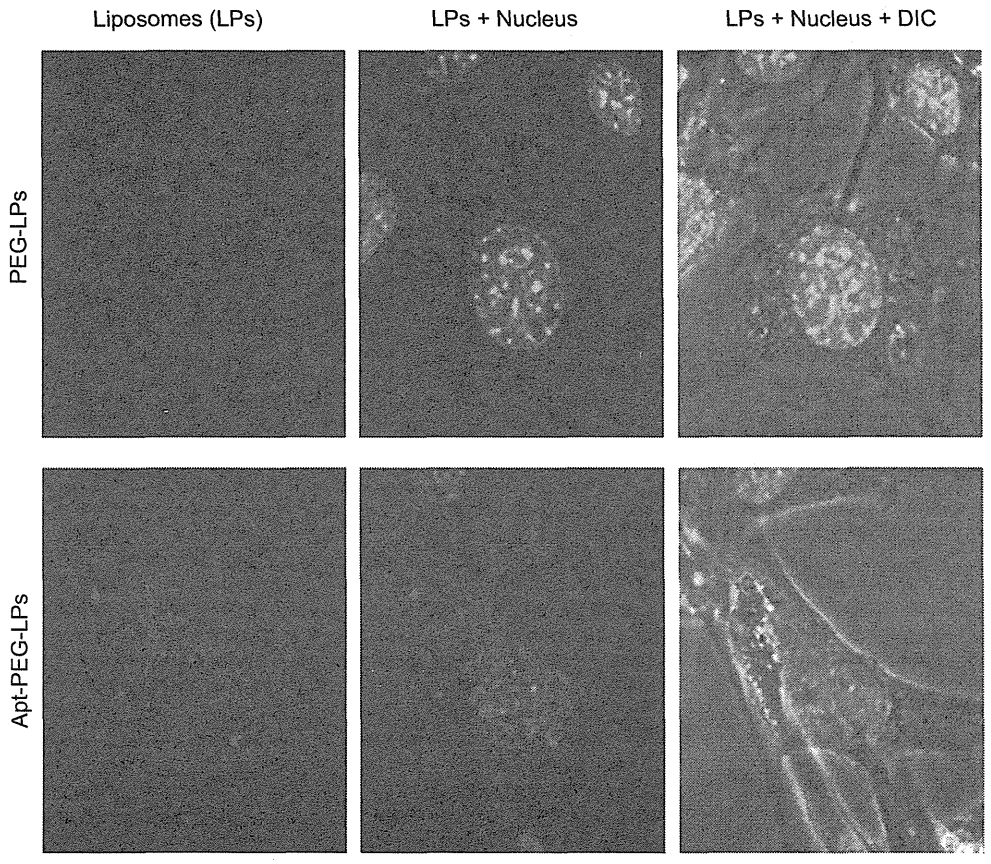

Fig. 3. Qualitative CLSM cellular uptake assay of Apt-PEG-LPs. SM-ECs, 200,000/35 mm glass bottom dish were treated with 5 mol% of the total lipid of Apt-PEG-LPs or PEG-LPs for 3 h at 37 °C. PEG-LPs and Apt-PEG-LPs containing Rhodamine incubated with SM-ECs for 3 h at 37 °C. Nuclei were stained with Hoechst 33342.

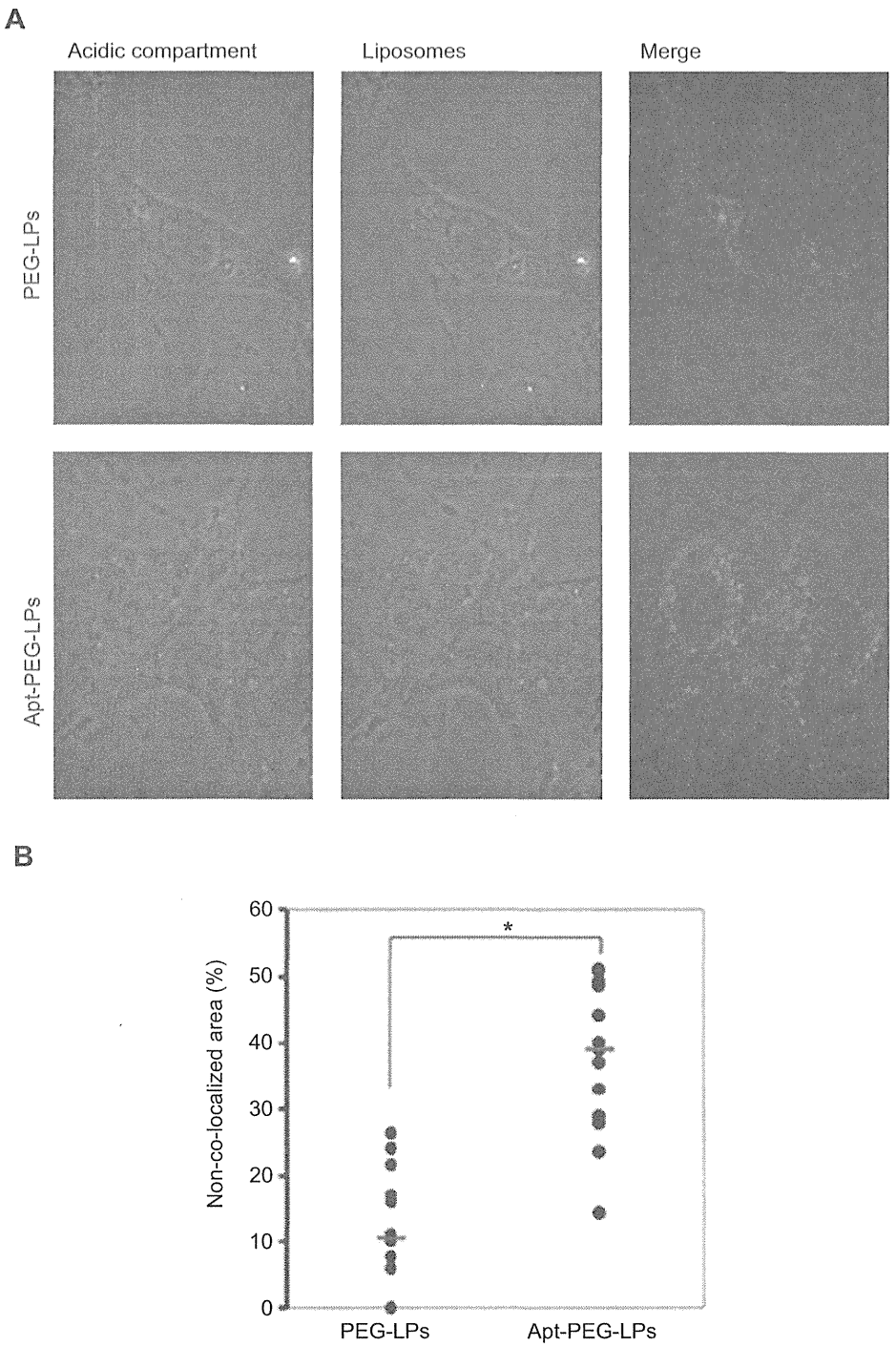

Fig. 4. Intracellular trafficking of Apt-PEG-LPs. (A) SM-ECs, 200,000/35 mm glass bottom were treated with 5 mol% of the total lipid of Apt-PEG-LPs or PEG-LPs for 3 h at 37 °C. PEG-LPs and Apt-PEG-LPs containing Rhodamine incubated with SM-ECs for 3 h at 37 °C. Cells were stained with Green Lysotracker, and nuclei were stained with Hoechst 33342 for 30 min. (B) Percent, % noncolocalize area of Apt-PEG-LPs. Image prosoftware were used to count the pixels corresponding to the Apt-PEG-LPs and unmodified PEG-LPs. Statistical analysis of different Apt-PEG-LPs v's PEG-LPs noncolocalized area was performed by unpaired student'*t* test ($n = 5$), $^*P < 0.05$, significant.

irrespective of whether other inhibitors had any influence on the uptake process (Fig. 6A). To further verify this conclusion, a quantitative analysis of uptake inhibition using different inhibitors was performed, as described above. The results indicated that the entry route followed by Apt-PEG-LPs was the same and the uptake was largely inhibited by sucrose (Fig. 6B).

3.7. In vivo targeting ability of AraHH001 modified liposomes by CLSM observation

Finally, we investigated the *in vivo* targeting ability of the Apt-PEG-LPs. We speculated that the nucleic acid moiety of the Apt-PEG_{2000}-DSPE might be recognized by immune cells due to the

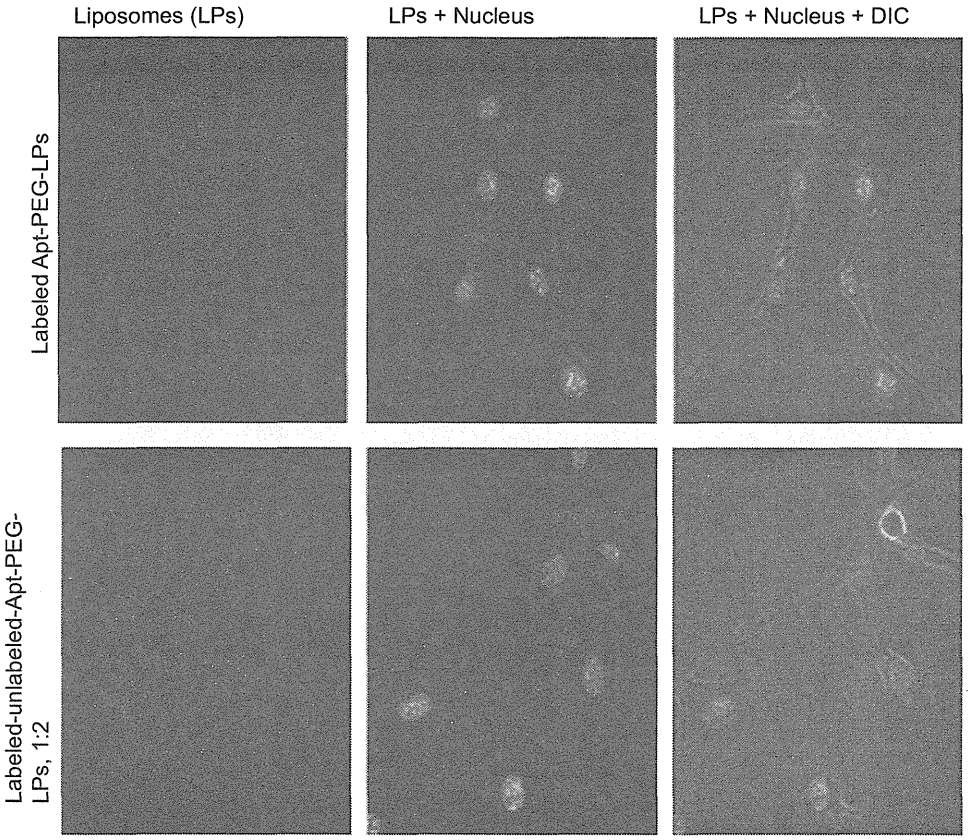

Fig. 5. Competition of cellular uptake with excess unlabeled Apt-PEG-LPs. SM-ECs, 2.0 × 10^5/35 mm glass bottom dish was treated with labeled or 1:2 mixture of Rhodamine labeled–unlabeled 5 mol% of the total lipid of Apt-PEG-LPs for 3 h at 37 °C. Nuclei were stained with Hoechst 33342 for 30 min.

presence of negatively charged phosphodiester groups, and would consequently be excreted from the liver and spleen in which immune cells including macrophages and lymphocytes would also be excreted. To circumvent such non-specific clearance, the Apt-PEG-LPs were modified with PEG_{5000}-DSPE (Apt/PEG_{5000}-LPs). Fluorescence labeled-Apt/PEG_{5000}-LPs were systemically injected into the human renal cell carcinoma (OS-RC-2 cells) bearing mice, and the tumor tissue was then observed by whole mounting CLSM 6 h after the administration. We previously reported that free AraHH001 binds to TECs derived from OS-RC-2 cells [37]. As the Apt-PEG_{2000}-DSPE was increased, the extent of co-localization of the LPs with tumor vessels was increased. When 5 mol% Apt-PEG-DSPE was incorporated, almost all of the LPs were observed in tumor vessels (Fig. 7). On the other hand, LPs modified with 1 or 2.5 mol% Apt-PEG_{2000}-DSPE were spread within the tumor xenograft. As to normal organs, the Apt-PEG-LPs were highly accumulated in the liver and spleen, but not in the heart, in which the target protein of AraHH001, troponin T, is expressed (Fig. S1).

To evaluate the selectivity of the Apt-PEG_{2000}-DSPE modified LPs for the tumor vasculature, we next quantified the ratio of co-localization by pixel counting. The percentage of yellow pixels to the total number of red pixels was defined as a co-localization ratio with TECs. The co-localization of the Apt/PEG_{5000}-LPs with tumor vessels was compared with the Apt-LPs and only PEG-LPs. The PEG-LPs were accumulated in tumor tissue via the enhanced permeability and retention (EPR) effect [39], and then diffused from tumor vessels because PEG did not bind specifically to cancer cells and TECs. Representative images are shown in (Fig. S2). In fact, the PEG-LPs were found to bare binded to TECs (3%), whereas the aptamer modified LPs were highly colocalized with the TECs (Apt-PEG-LPs 16%, Apt/PEG_{5000}-LPs 25%) (Fig. 8).

4. Discussion

Recently, our collaborative group isolated very pure tumor endothelial cells, in an attempt to better understand the effects of the tumor microenvironment on the properties of endothelial cells and showed they are different from normal endothelial cells. Additionally, tumor endothelial cells are cytogenetically abnormal. Thus, it can be assumed that cultured tumor endothelial cells are more relevant than normal endothelial cells in studies of tumor angiogenesis. It has been challenging to isolate and culture tumor endothelial cells because (i) endothelial cells are usually enmeshed in a complex type of tissue, consisting of vessel wall components, stromal cells, and tumor cells; and (ii) only a small fraction of cells within these tissues are endothelial cells. Our goal is Vascular targeting, an attractive strategy that takes into account phenotype changes on the surface of endothelial cells under pathological conditions, such as angiogenesis and inflammation [32–36]. To achieve our goal, we first isolated a mTEC-specific DNA aptamer AraHH001 which confirms the selective expression not only on the surface of primary cultured mouse tumor endothelial cells of different origin, even it was expressed on the surface of primary cultured human tumor endothelial cells. Additionally, this isolated

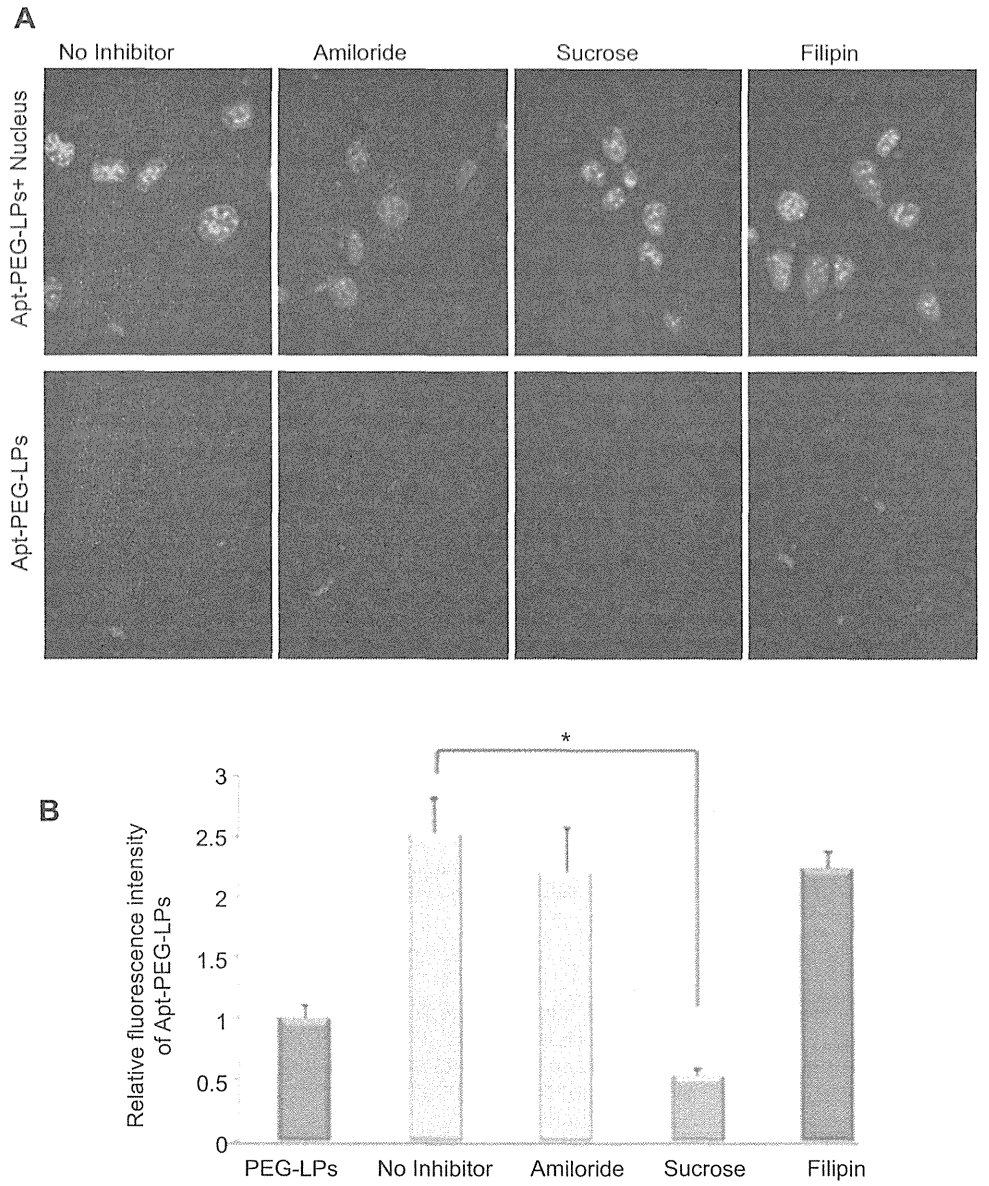

Fig. 6. Uptake inhibition assay of Apt-PEG-LPs by different inhibitors. Cells were pre-incubated in the absence or presence of 1 mM amiloride, 10 min, 1 mg/mL Filipin, 30 min, 0.25 M sucrose, 30 min SM-ECs, (A) In CLSM study, 200000 pre-incubated cells per 5 mm glass bottom were treated with 5 mol% of the total lipid of Apt-PEG-LPs for 1 h at 37 °C. Cells treated with only Apt-PEG-LPs used as a control. Apt-PEG-LPs containing Rhodamine, and nuclei were stained with Hoechst 33342 for 30 min (B) Quantitative inhibition of uptake of Apt-PEG-LPs were investigated using the above procedure. Here 4×10^4 cells/24-well were used. After treatment 1× lysis buffer (Promega) was used to lysate the cells. Finally, the quantification of fluorescent intensity was measured by spectrofluorometer. Data shown as mean ± SD, $n = 4$.

aptamer ligand has a tendency to be internalized by cells very well [37]. Therefore, our intent was to apply this promising, DNA aptamer ligand in the construction of an Apt-PEG-LPs nano-carrier system for further internalization studies to confirm its ability to target mTECs, thus leading to the development of a drug delivery system.

Both DNA and RNA aptamers for several different targets have been successfully screened in last two decades, and this approach is now considered to be the first choice probe and ligand for the development of future targeting nano-medicine [8–12]. We prepared an aptamer modified PEGylated nano-carrier system by attaching the 5-thioated aptamer ligand AraHH001 at the maleimide-PEG terminus on the liposomes. First, we cleave the AraHH001-S–S bond to produce an AraHH001-SH bond by treatment with a reducing agent TCEP. A NAP-column was used to purify the AraHH001-SH which was further used for the conjugation with maleimide-PEG_{2000}-DSPE. Dialysis (MWCO 3500–5000) was performed until the pure aptamer-lipid conjugation was obtained. MALDI-TOF spectroscopy was employed to check the purity. Finally, the UV-visible spectroscopy was applied to measure the aptamer-lipid concentration. In this study, we attached our aptamer ligands to the distal ends of PEG chains. This would be more effective than directly attaching ligands to the surface of PEG-containing liposomes because, PEG chains interfere with both the coupling of ligands to the lipid bilayer and the interaction of these ligands with the intended biological targets. These ligands coupled to the

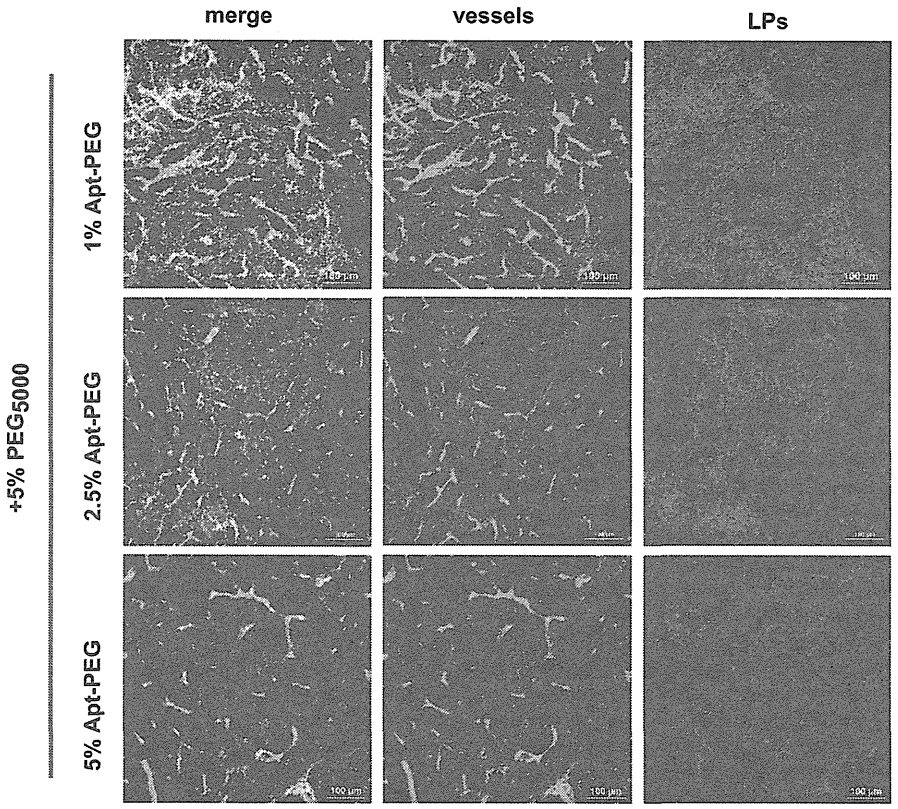

Fig. 7. Intratumoral distribution of the Aptamer modified LPs. The fluorescently labeled-LPs were injected into the tumor-bearing mice at a lipid dose of 750 nmol, and tumor was collected 6 h after the injection. Prior to collection tumor vessels were visualized by FITC-labeled isolectin. Green and red dots indicate vessels and LPs, respectively.

PEG terminus do not cause any interference with the binding of ligands to their respective recognition molecules [40].

First, we assessed an aptamer-decorated PEGylated nano-carrier system and found that is showed a significant level of cellular uptake compared to the unmodified PEGylated nano-carrier system in mTECs (Fig. 2). This result also indicated that the targeted aptamer first recognized the cellular surface of the target molecule and was then internalized. Next, to visualize the extent of enhanced cellular uptake we carried out an *in vitro* qualitative CLSM uptake study (Fig. 3). The Rhodamine labeled Apt-PEG-LPs were found to have a very higher internalization capacity in mTECs compared to unmodified PEG-LPs. Therefore, the above results suggest that modifying the PEGylated liposomes with the targeting ligand is essential for the association, and the internalization of the nano-carrier system into mTECs. At the same time, due to the steric repulsion of the PEG polymer to unmodified PEGylated Liposomes, the extent of association to the target mTECs is decreased, and thus the uptake efficacy was lower.

We next concentrated on a crucial step, i.e., addressing the distribution of ligand modified LPs, and their capacity to escape from endosomes. There is a very common but important phenomena called endosomal degradation that might interfere with the delivery of drugs or genes of a targeted carrier mediated nano-carrier to a specific site. To clarify this issue we carried out an intracellular trafficking experiment in which the uptake of Rhodamine labeled Apt-PEG-LPs was evaluated lyosotracker green as an intracellular marker. A CLSM study of intracellular trafficking showed there some Apt-PEG-LPs were co-localized with lysotracker green as visualized as yellow (in the web version) (Fig. 4A). However, some remaining Apt-PEG-LPs that were un-colocalized but remained intact inside the cytoplasm could be observed (Fig. 4A). Whereas, PEG-LPs were not taken up substantially and therefore, it was difficult to determine whether they were colocalized or not. We then applied image pro software to count different pixel areas and thus determine the co-localized, and non-co-localized areas of both the Apt-PEG-LPs and PEG-LPs. From the analysis of pixel counts it was found that the concentration of non-colocalized Apt-PEG-LPs was higher, than PEG-LPs. Next, we

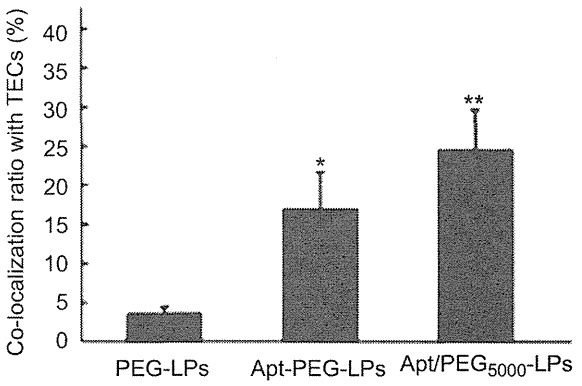

Fig. 8. Investigation of the targeting efficiency to tumor vessels of the aptamer-modified LPs. Co-localization ratio with TECs were calculated by pixel counting the pictures (the representative images were indicated in Fig. S2) and calculating using the following equation; Co-localization ratio with TECs = (yellow pixels)/(red + yellow pixels). Data represents mean ± SD. Statistical analysis was performed one way ANOVA followed by SNK test; $^{*}P < 0.05$, $^{**}P < 0.01$ v's PEG-LPs, $n = 3$.

calculated the percent of non-colocalized uptake for the Rhodamine signal of the Apt-PEG-LPs and PEG-LPs. Approximate 39% the Apt-PEG-LPs were non-colocalized, which was statistically significant as compared with the per sent uptake for the PEG-LPs (Fig. 4B).

We next explored the uptake mechanism responsible for the aptamer modified PEGylated nano-carrier system. Since we recently developed a DNA aptamer AraHH001 that specifically targets mTECs, it was applied in this project to develop a ligand based liposomal nano-carrier system. Our plan was to use unlabeled Apt-PEG-LPs to block the receptors (details can be found in the experimental section) from accessing the labeled Apt-PEG-LPs in a competition experiment. Only labeled Apt-PEG-LPs were used as a control to compare. The CLSM results suggested that, although not complete, that the inhibition of uptake of aptamer targeted nano-carrier occurred. This result, provides evidence to indicate that the uptake of Apt-PEG-LPs is a receptor mediated process (Fig. 5). We next investigated the specific pathway responsible for receptor mediated endocytosis, by using different receptor mediated endocytic inhibitors. The CLSM experimental results showed that our aptamer modified PEGylated nano-carrier system follows clathrin mediated endocytosis.

Receptor-mediated endocytosis is generally considered to be a very promising, and widely accepted approach to drug targeting. Most of the currently used ligands are internalized by clathrin-mediated endocytosis, consistent with our findings [38,41]. Interestingly, our findings suggested that the newly developed aptamer ligand based PEGylated nano-carrier system exhibits a higher endosomal escaping capacity, although the exact reason for this is not currently clear. It is well known that poor intracellular trafficking is often associated with clathrin mediated endocytosis. Molecules entering a cell via this pathway rapidly experience a drop in pH from neutral pH 5.9 to 6.0 in the lumen of early endosomes with a further reduction to pH 5 during progression from late endosomes to lysosomes, where ligands fused with it, eventually resulting in degradation [42]. However, it was previously reported that most ligands follow the clathrin mediated receptor specific endocytosis [43].

To date, only a few reports showing that aptamer modified liposomes are applicable to *in vivo* situations have appeared [24,25]. To our knowledge, this is the first report to demonstrate the specific delivery of TECs using aptamer modified liposomes. The Apt-PEG-LPs and Apt/PEG_{5000}-LPs were selectively bound to TECs, but not cancer cells after systemic injection. It is noteworthy that the PEG_{5000}-DSPE modification appeared to facilitate the TEC delivery of Apt-PEG-LPs (Fig. 8). This can be attributed to the fact that the PEGylation partially covered the aptamers, and hence prevented them from being recognized by immune cells, such as macrophages. In previous reports, oligo nucleic acids were taken up via scavenger receptors [44], which are expressed in macrophages [45]. Accordingly, PEG_{5000}-DSPE modification appears to improve the pharmacokinetics of Apt-PEG-LPs, and therefore Apt/PEG_{5000}-LPs might be able to accumulate at much higher levels in tumor vessels than Apt-PEG-LPs.

5. Conclusion

We report on the development of an AraHH001 aptamer modified PEGylated liposomal nanocarrier system for targeted delivery toward tumor vasculature *in vitro* and *in vivo*. Our system enhanced specific cellular uptake in mTECs and has the capacity, to a certain extent, to escape from endosomes, a process that might be useful for future targeting drug delivery to tumor endothelial cells. We further confirmed that our Apt-PEG-LPs follow receptor specific and clathrin mediated endocytosis. Apt-PEG-LPs and Apt/PEG_{5000}-LPs showed higher accumulation on tumor vasculature *in vivo*. The findings of our system complete all the criteria that is primarily essential for a ligand based active drug delivery system, and would be very useful for the treatment of cancer and many related diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2014.04.087.

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