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Title	An aptamer ligand based liposomal nanocarrier system that targets tumor endothelial cells
Author(s)	Ara, Mst Naznin; Matsuda, Takashi; Hyodo, Mamoru et al.
Citation	Biomaterials, 35(25), 7110-7120 https://doi.org/10.1016/j.biomaterials.2014.04.087
Issue Date	2014-08
Doc URL	https://hdl.handle.net/2115/57445
Type	journal article
File Information	WoS_66920_Sakurai.pdf

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An aptamer ligand based liposomal nanocarrier system that targets tumor endothelial cells

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ABSTRACT

The objective of this study was to construct our recently developed aptamer-modified targeted liposome nano-carrier (Apt-PEG-LPs) system to target primary cultured mouse tumor endothelial cells (mTEC), both *in vitro* and *in vivo*. We first synthesized an aptamer-polyethylene glycol 2000-distearoyl phosphoethanolamine (Apt-PEG_{2000}-DSPE). The conjugation of the Apt-PEG_{2000}-DSPE was confirmed by MALDI-TOF mass spectroscopy. A lipid hydration method was used to prepare Apt-PEG-LPs, in which the the outer surface of the PEG-spacer was decorated with the aptamer. Apt-PEG-LPs were significantly taken up by mTECs. Cellular uptake capacity was observed both quantitatively and qualitatively using spectrofluorometry, and confocal laser scanning microscopy (CLSM), respectively. In examining the extent of localization of aptamer-modified liposomes that entered the cells, approximately 39% of the Apt-PEG-LPs were not co-localized with lysotracker, indicating that they had escaped from endosomes. The uptake route involved a receptor mediated pathway, followed by clathrin mediated endocytosis. This Apt-PEG-LP was also applied for *in vivo* research whether this system could target tumor endothelial cells. Apt-PEG-LP and PEG_{5000}-DSPE modified Apt-PEG-LP (Apt/PEG_{5000}-LP) were investigated by human renal cell carcinoma (OS-RC-2 cells) inoculating mice using CLSM. Apt-PEG-LP and Apt/PEG_{5000}-LP showed higher accumulation on tumor vasculature comapred to PEG-LP and the co-localization efficacy of Apt-PEG-LP and Apt/PEG_{5000}-LP on TEC were quantified 16% and 25% respectively, which was also better than PEG-LP (3%). The findings suggest that this system is considerable promise for targeting tumor endothelial cells to deliver drugs or genes *in vitro and in vivo.*

Key words: Targeted delivery, Aptamer-liposomes, Endocytosis, Intracellular uptake

Abbreviations: LPs, Liposomes; Apt, Aptamer; PEG, maleimide-PEG_{2000}-DSPE; PEG-LPs, maleimide-PEG_{2000}-DSPE modified liposomes; Apt-PEG-LPs, Aptamer modified maleimide-PEG_{2000}-DSPE liposomes; mTECs: Primary cultured mouse tumor endothelial cells

1. Introduction

A continuous affordable, but still greater challenge remains in nano-medicine in terms of cancer diagnosis, and therapy designed to deliver imaging agents or chemotherapeutic drugs to cells in a specific and selective manner [1]. The successful delivery of cytotoxic drugs via passive or active targeting is an important issue in the design and construction of new and improved targeting drug delivery systems. Small molecules such as peptides, as well as antibodies have been widely used targeting agents, and has enjoyed some (but not sufficient) success, when incorporated with nano-materials. The resulting constructs are often dictated more by the materials used rather than the targeting agents. Researchers are currently attempting to develop more and more new types of therapy [2-5]. To meet these challenges, nucleic acid aptamers are now of great interest as new targeting small molecules. Aptamers are single stranded oligonucleotides, ssDNA or ssRNA molecules produced by SELEX [6, 7]. Cell-SELEX is a modified selection method against live cells [8]. Aptamers are very easy to reproduce, are low in cost, generally nontoxic, and have a low molecular weight (8-15 kDa). This single stranded DNA or RNA oligonucleotide can fold into well-defined 3D structures and bind to their target with a high affinity (μM to pM range) and specificity [9, 10].

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 avialable. 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 bio-distribution 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 alonger 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 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 tarfficking 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 mole% 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 30sec-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 wer 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 12000 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).

201

202 *2.6. Qualitative cellular uptake analysis Apt-PEG-LPs in mTECs by confocal laser scanning*
203 *microscopy (CLSM)*

204 For performing Confocal microscopy, 2×10^5 mTECs were seeded per 35-mm glass
205 bottom dish (Iwaki, Chiba, Japan) in 2 mL of culture medium 24 h before the experiment in a
206 37 °C incubator under an atmosphere of 5% CO_2, and in 95% humidity. On the next
207 experimental day, the medium was removed from the cells by aspiration and the cells were
208 then washed once with 2 mL of 1× PBS, and then incubated with 5 mole% of the total lipid of
209 PEG-LPs, or Apt-PEG-LPs in Kreb's buffer for 3 h at 37 °C. After 2.5 h of incubation, 20 µl
210 of Hoechst 33342 (1 mg/mL) was added to stain the nuclei and the suspension was re-
211 incubated for an additional 30 min. The medium was then removed and the cells were washed
212 twice with a 1 mL of 1× PBS supplemented with cholic acid (10 nM). Finally, 1 mL of Krebs
213 buffer was added and the cells were analyzed under confocal microscopy (A1 Confocal Laser
214 Microscope System, Olympus Instruments Inc., Tokyo, Japan).

215

216 *2.7. Intracellular trafficking of Apt-PEG-LPs in mTECs via Confocal laser scanning*
217 *Microscopy (CLSM)*

218 mTECs were seeded in 35 mm glass bottom dish with 2 mL of medium and incubated for
219 24 h. The cell density was 2×10^5 cells /glass bottom dish. On the next experimental day, the
220 cells were incubated with 5 mole% of the total lipid of Apt-PEG-LPs and PEG-LPs in Krebs
221 buffer for 3 h at 37 °C under an atmosphere with 5% CO_2 and in 95% humidity. The cells
222 were stained with LysoTracker green (DND-26) 1 (µg/ mL) for 30 min at 37 °C. After 2-3
223 washings with 1× PBS supplemented with 10 nM cholic acid, the cells were examined by
224 confocal laser scanning microscopy, as described above.

225

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 molar 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 mole% 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% CO2 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% CO2 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 lysss 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 12000 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. Qulititative Evaluation of the in vivo intratumoral localization of systemically injected Apt-PEG-LPs.

Human renal cell carcinoma, 1 x 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 clearacne 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 mole% 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 5mole% 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 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 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 acompetition 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, 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 presence of negatively charged phosphordiester 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 mole% 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 mole% 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 pearmeablity 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%), wheras the aptamer modified LPs were highly co-localized 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

399 into account phenotype changes on the surface of endothelial cells under pathological
400 conditions, such as angiogenesis and inflammation [32-36]. To achieve our goal, we first
401 isolated a mTEC-specific DNA aptamer AraHH001 which confirms the selective expression
402 not only on the surface of primary cultured mouse tumor endothelial cells of different origin,
403 even it was expressed on the surface of primary cultured human tumor endothelial cells.
404 Additionally, this isolated aptamer ligand has a tendency to be internalized by cells very well
405 [37]. Therefore, our intent was to apply this promising, DNA aptamer ligand in the
406 construction of an Apt-PEG-LPs nano-carrier system for further internalization studies to
407 confirm its ability to target mTECs, thus leading to the development of a drug delivery
408 system.
409 Both DNA and RNA aptamers for several different targets have been successfully screened in
410 last two decades, and this approach is now considered to be the first choice probe and ligand
411 for the development of future targeting nano-medicine [8-12]. We prepared an aptamer
412 modified PEGylated nano-carrier system by attaching the 5- thioated aptamer ligand
413 AraHH001 at the maleimide-PEG terminus on the liposomes. First, we cleave the
414 AraHH001-S-S bond to prpduce an AraHH001-SH bond by treatment with a reducing agent
415 TCEP. A NAP-column was used to purify the AraHH001-SH which was further used for the
416 conjugation with maleimide-PEG_{2000}-DSPE. Dialysis (MWCO 3500-5000) was performed
417 until the pure aptamer-lipid conjugation was obtained. MALDI-TOF spectroscopy was
418 employed to check the purity. Finally, the UV-visible spectroscopy was applied to measure
419 the aptamer-lipid concentration. In this study, we attached our aptamer ligands to the distal
420 ends of PEG chains. This would be more effective than directly attaching ligands to the
421 surface of PEG-containing liposomes because, PEG chains interfere with both the coupling of
422 ligands to the lipid bilayer and the interaction of these ligands with the intended biological

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

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

437 We next concentrated on a crucial step, i.e., addressing the distribution of ligand modified
438 LPs, and their capacity to escape from endosomes. There is a very common but important
439 phenomena called endosomal degradation that might interfere with the delivery of drugs or
440 genes of a targeted carrier mediated nano-carrier to a specific site. To clarify this issue we
441 carried out an intracellular trafficking experiment in which the uptake of Rhodamine labeled
442 Apt-PEG-LPs was evaluated lyosotracker green as an intracellular marker. A CLSM study of
443 intracellular trafficking showed there some Apt-PEG-LPs were co-localized with lysotracker
444 green as visualized as yellow (Fig. 4A). However, some remaining Apt-PEG-LPs that were
445 un-colocalized but remained intact inside the cytoplasm could be observed (Fig. 4A).
446 Whereas, PEG-LPs were not taken up substantially and therefore, it was difficult to
447 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 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.

Acknowledgments

This study was supported by grants from the Special Education and Research Expenses of the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was also supported by Grant-In-Aid for Young Scientists (B, 11018330) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors also thank Dr. Milton S. Feather for his advice in writing the English manuscript.

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Figure legends

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.

Fig.2. Quantitative cellular uptake assay of Apt-PEG-LPs. SM-ECs, 4×10^4/24-well were treated with 5 mole% 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.

Fig.3. Qualitative CLSM cellular uptake assay of Apt-PEG-LPs. SM-ECs, 200000/35mm glass bottom dish were treated with 5 mole% 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, 200000/35 mm glass bottom were treated with 5 mole% 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.

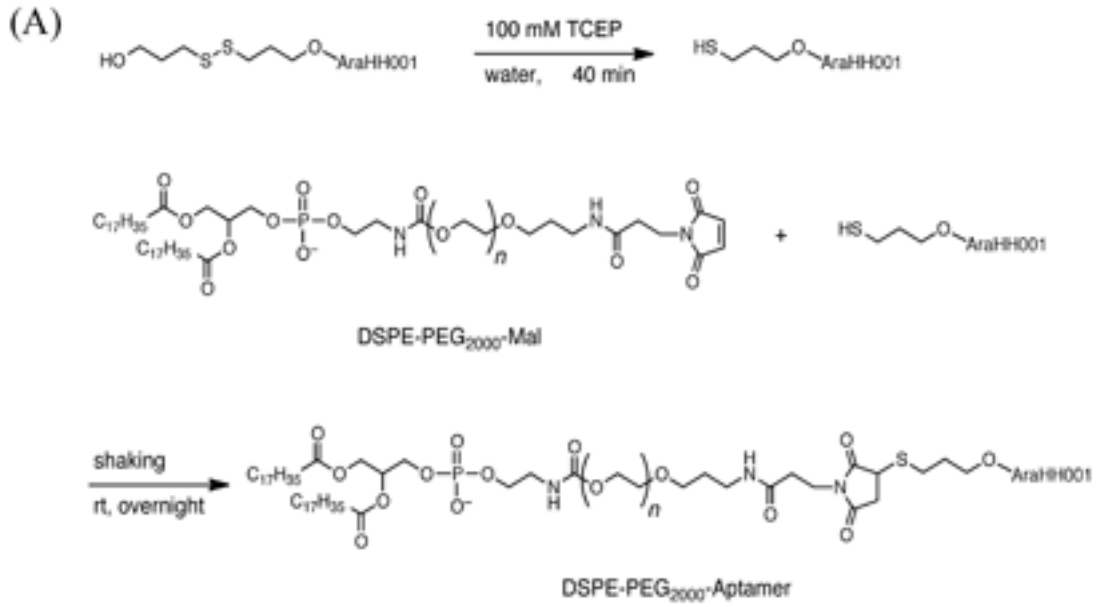
Fig.5. Competition of cellular uptake with excess unlabeled Apt-PEG-LPs. SM-ECs, 200000/35 mm glass bottom dish was treated with labeled or labeled-unlabeled 5mol% of the total lipid of Apt-PEG-LPs for 3 h at 37 °C. Apt-PEG-LPs that contained Rhodamine, and nuclei were stained with Hoechst 33342 for 30 mins. (B) Percent, % noncolocalize area of Apt-PEG-LPs. Image prosoftware were used to count pixels of 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.

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, 1mg/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 mole% 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) Quantitaive inhibition of uptake of Apt-PEG-LPs were investigated using the above procedure. Here $4x10^4$ 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

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 visalized by FITC-labled isoletin. Green and red dots indicate vessels and LPs, respectively.

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 caliculating 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.

Fig. 1.


(A)
100 mM TCEP
water, 40 min
AraHH001
DSPE-PEG2000-Mal
shaking
rt, overnight
DSPE-PEG2000-Aptamer
TCEP = tris(2-carboxyethyl)phosphine hydrochloride, efficient reducing agent for disulfide linkage

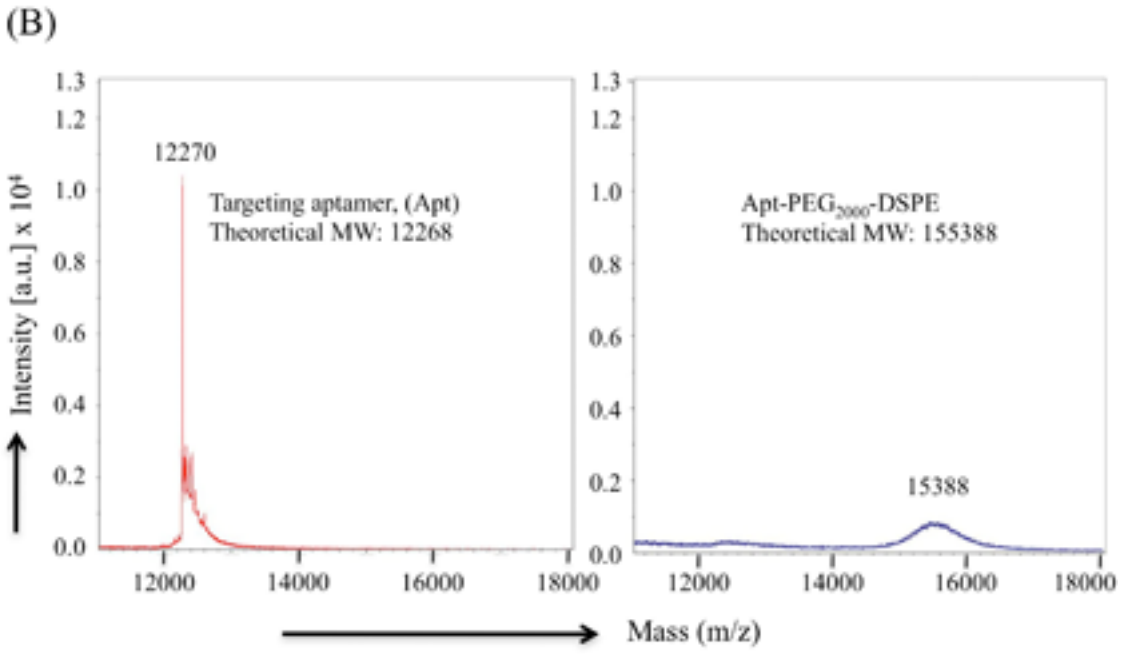
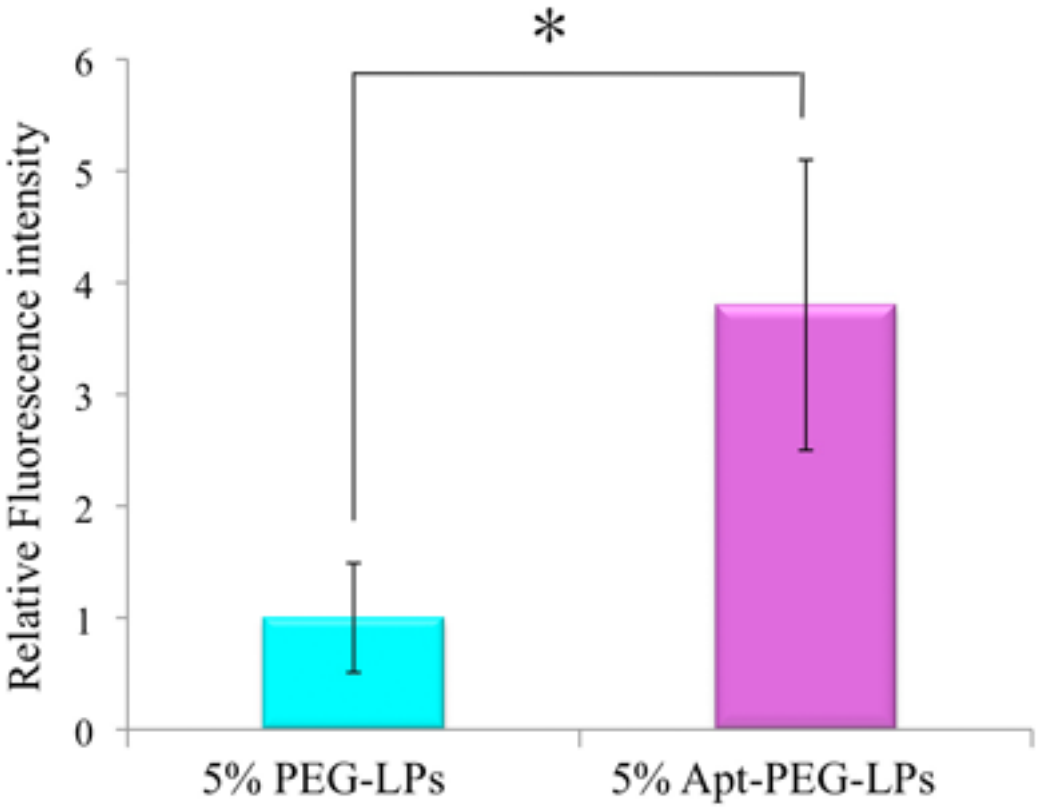

(B)
12270
Targeting aptamer, (Apt)
Theoretical MW: 12268
Apt-PEG2000-DSPE
Theoretical MW: 155388
15388
Intensity [a.u.] x 10⁴
Mass (m/z)

Fig. 2.



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676

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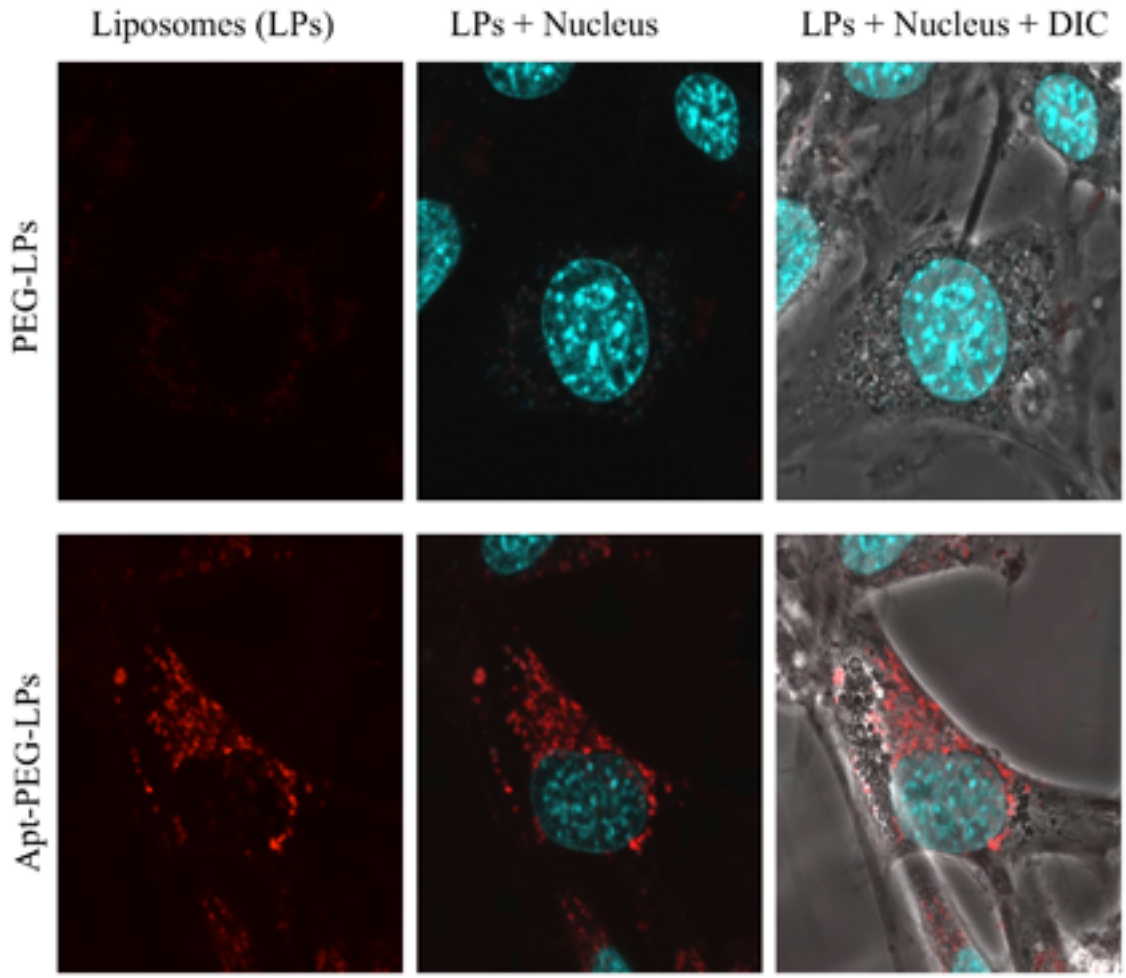
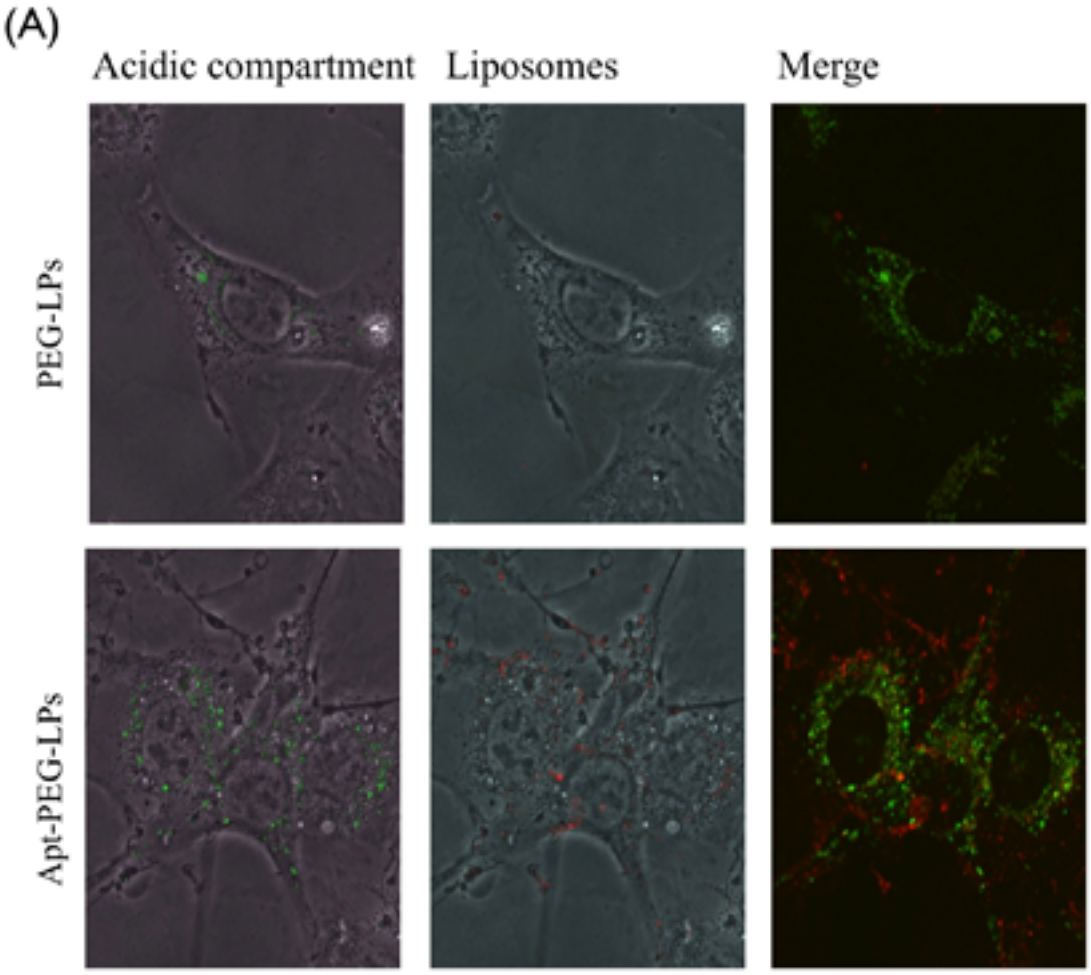
Fig. 3.


Liposomes (LPs)
LPs + Nucleus
LPs + Nucleus + DIC
PEG-LPs
Apt-PEG-LPs

678

679

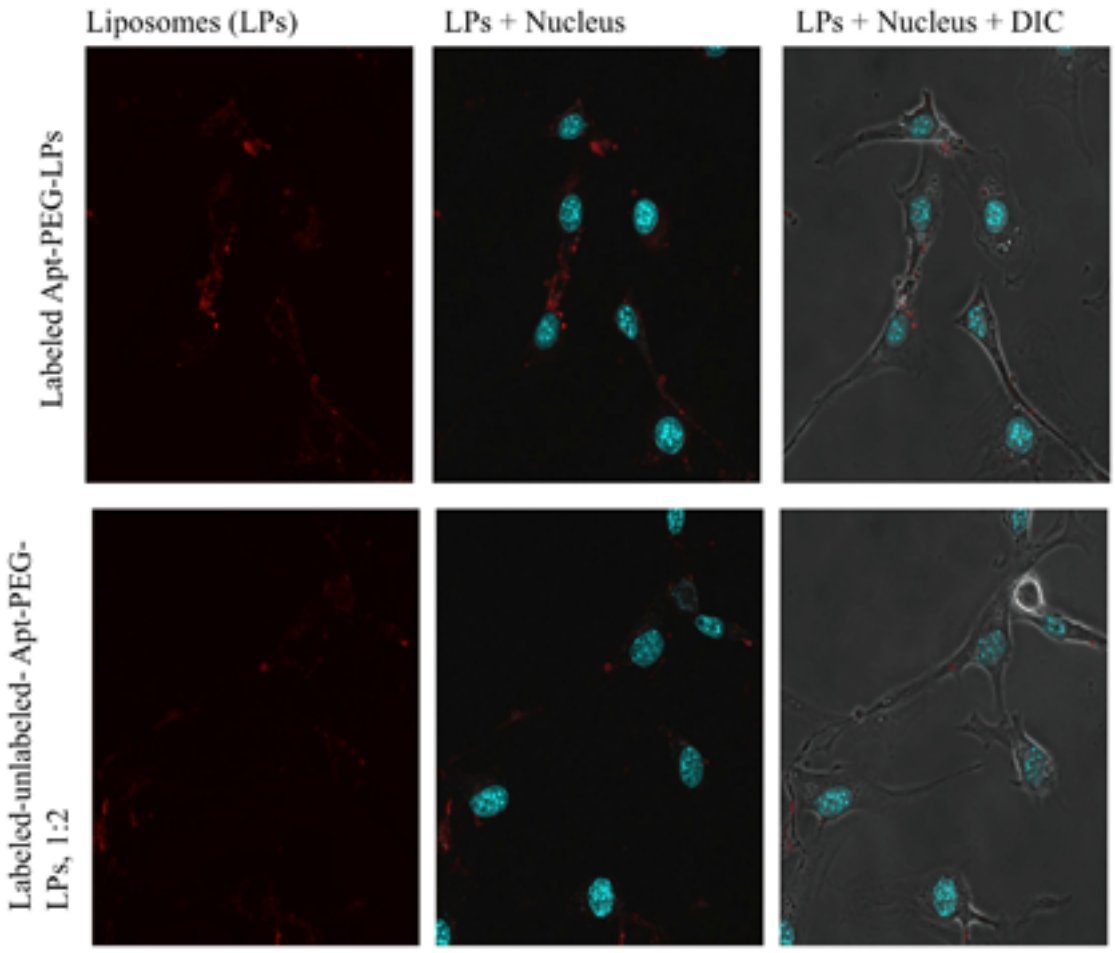
Fig. 4.


(A)
Acidic compartment
Liposomes
Merge
PEG-LPs
Apt-PEG-LPs
(B)
Non-co-localized area (%)
60
50
40
30
20
10
0
*
PEG-LPs
Apt- PEG-LPs

680

681

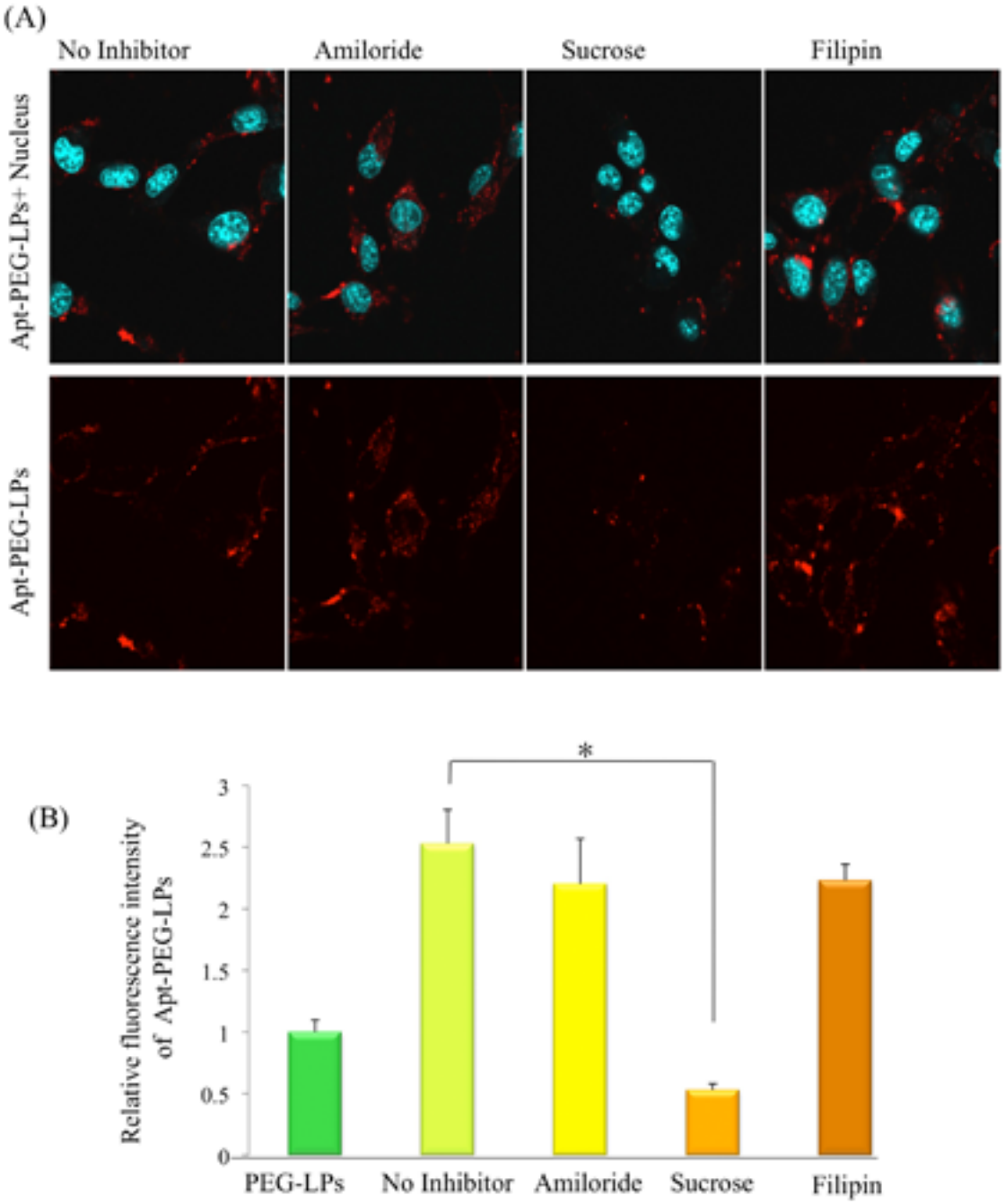
Fig. 5.


Liposomes (LPs)
LPs + Nucleus
LPs + Nucleus + DIC
Labeled Apt-PEG-LPs
Labeled-unlabeled- Apt-PEG-LPs, 1:2

682

683

Fig. 6.



684

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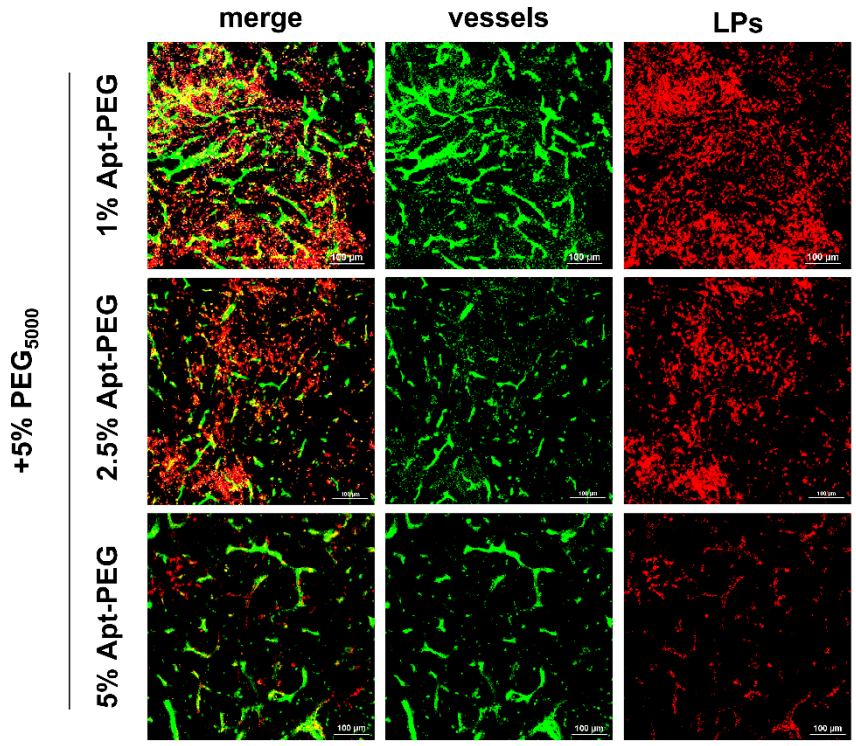
686

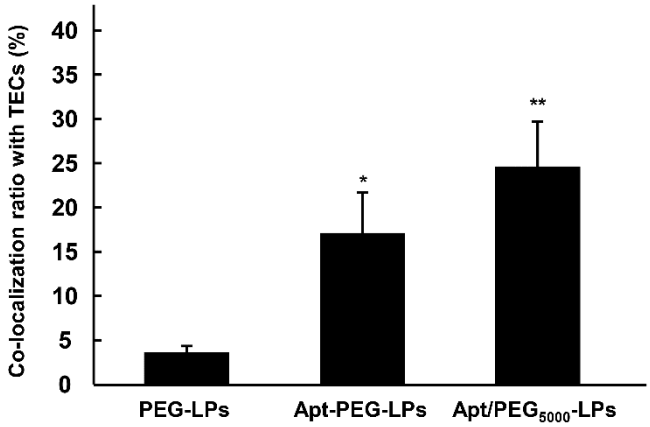
687 **Fig. 7**



688

689

690 **Fig. 8**



691