Dual-ligand modification of PEGylated liposomes shows better cell selectivity and efficient gene delivery

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Abstract
The objective of this study was to develop an efficient dual-ligand based PEGylated liposomal delivery system that had target specificity as well as properties that would enhance cellular uptake. PEGylated liposomes (PEG-LP) were prepared by the lipid film hydration method by adding distearoyl phosphoethanolamine-polyethylene-glycol-2000 conjugate (DSPE-PEG2000) to a lipid mixture. The cyclic RGD (Arg-Gly-Asp) peptide, a specific ligand with affinity for Integrin αvβ3 was coupled to the distal end of the PEG on the PEG-LP (RGD-PEG-LP). Stearylated octaarginine (STR-R8) was incorporated on the surface of the RGD-PEG-LP as dual-ligand (R8/RGD-PEG-LP) that functions as a cell penetrating peptide (CPP). RGD-PEG-LP and R8/RGD-PEG-LP were preferentially taken up by caveolae-mediated and clathrin-mediated endocytosis pathways, respectively. Compared to PEG-LP, R8/RGD-PEG-LP showed an enhanced cellular uptake as well as a higher transfection efficiency in Integrin αvβ3 expressing cells. However, the amount of cellular uptake or gene expression by the single ligand versions was negligible, even in Integrin αvβ3 expressing cells. No remarkable difference in cellular uptake or gene expression was observed for cells in which the expression of targeted receptors was absent. It can be concluded that dual-ligand modified PEG-LP possesses a strong capability for the efficient internalization of PEG-LP and consequently would be an effective tool for the targeted delivery of macromolecules or chemotherapeutics through accelerated cellular uptake.

Key words
Targeted delivery, dual-ligand, PEGylated liposome, RGD motif, cell penetrating peptide, gene expression
1. Introduction

Liposomes are now well-recognized drug delivery vehicles that can be used in cancer therapy. They have been shown to enhance the therapeutic activity of several anticancer drugs [1]. PEGylated liposomes (PEG-LPs), also referred to as stealth liposomes (SLs), have the ability to passively accumulate in tumor tissues through Enhanced Permeability and Retention (EPR) effects [2]. However, PEGylation reduces the extent of interactions of LPs with target cells, resulting in improper cellular uptake as well as poor endosomal escape, a factor that ultimately leads to a significant loss of the pharmacological effect of a drug. The negative effect of PEGylation on the target site is called the PEG dilemma, which hampers the practical application of drugs or other macromolecules, when the use of LPs is being considered [3, 4]. To overcome this problem, various ligands such as transferrin [5], folic acid [6], peptides [7,8] or antibodies [9] are added at the terminal ends of PEG moieties to produce active targeting PEG-LPs. Anticancer drugs that are encapsulated within ligand modified PEG-LPs demonstrate increased binding, improved cytotoxicity and, in many cases, improved therapeutic efficacy, compared with PEG-LPs [10-11]. However, interactions between the specific ligand and the target molecule facilitate the receptor mediated endocytosis of liposomes [12], which is a saturation phenomenon that again limits the cellular uptake of PEG-LPs. Therefore, the addition of a specific ligand to the PEG-LPs could not provide satisfactory enhancement of cellular association as well as better therapeutic effects of delivered cargos.

Cell-penetrating peptides (CPPs), short peptide sequences have recently been used for drug or gene delivery [13]. They have the ability to enter cells, either alone or in conjugation with small molecules or bulky cargos such as peptides, proteins, oligonucleotides, plasmid DNA (pDNA) or liposomes. As a CPP, stearylated octaarginine (STR-R8) modified liposomes were developed by our group, and were found to be internalized by target cells and to efficiently deliver drugs, pDNA, small interfering RNA (siRNA) and proteins in vitro [14-16]. Although CPPs are able to deliver a therapeutic moiety to target cells, their non-specific affinity towards cell lines makes them inefficient in terms of designing an effective targeted delivery system for biodistribution as well as for systemic administration. To overcome these impediments, it would be desirable to establish a suitable protocol based on a “Dual-ligand system” considering the specific ligand and CPP that might result in successful targeted cancer therapy.

In this study, we report on the design of a dual-ligand based PEG-LP in which a specific receptor targeted peptide was attached to the distal end of PEG molecules of a PEG-LP after which, the CPP ligand was attached to the surface of the
liposome as a cationic ligand. In order to ensure the synergistic effect of the preparation in the systemic circulation, the CPP should remain inactive and free from opsonins but, after its arrival at the target site, CPP would exert efficiency to internalize the liposomes into the target cell. To confirm the dual-ligand concept using a combination of CPP and a specific ligand, we employed a RGD motif peptide (Arginine-Glycine-Aspartic acid) as a specific ligand for Integrin αvβ3 and STR-R8 as a CPP ligand. The RGD peptide is the adhesion motif of extracellular matrix proteins for the various types of integrins [17,18]. Integrin αvβ3 is considered to be an important target in the development of new anticancer strategies because of its higher level of expression on the surface of different cancer cells e.g. gliomas [19], melanomas [20], ovarian carcinomas [21], as well as tumor-associated endothelial cells [22]. It has been reported that Integrin αvβ3 plays an important role in angiogenesis [22], metastasis [23] and in resistance to radiotherapy [24]. It should also be noted that the binding affinity of the cyclic RGD peptide for Integrin αvβ3 is reported to be 1000 times greater than that for the linear RGD peptide [25]. The dual-ligand PEG-LP fabricated with cyclic RGD peptide (cRGDFK) and STR-R8 ligands was designed to target Integrin αvβ3 expressing cells. We evaluated the cellular uptake efficiency as well as the pharmacological application of the dual-ligand modified PEGylated liposome and the results were compared with corresponding data for the single-ligand version.
2. Materials and methods

2.1. Materials

1,2-dioleoyl-3-trimethylammonium-propane (chlordide salt) (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), Egg phosphatidylcholine (EPC), Cholesterol, N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (rhodamine-DOPE), 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3-(N-succinimidylxyloglutaryl) aminopropyl, polyethyleneglycol [PEG] 2000-carbamyl distearoylphosphatidyl-ethanolamine (NHS-PEG-DSPE) were purchased from the NOF Corporation, Japan. cRGDfK peptide and Stearylated octaarginine (STR-R8) were purchased from Peptides International, Inc. (Louisville, KY, USA) and Polypeptide Laboratories (San Diego, CA, USA) respectively. Heparin, Sucrose and Hoechst 33342 were received from Wako Pure Chemical Industries (Osaka, Japan). Endothelial Cell Basal Medium (EBM-2) and bullet kit were purchased from Lonza (Walkersville, MD, USA). Poly aspartic acid, Triton X-100, amiloride, filipin III from Strepotmyces filipinensis were obtained from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). The anti-human Integrin \( \alpha_v \beta_3 \) antibody and Alexa Fluor 488 labeled goat anti-mouse IgG were purchased from R & D systems Inc. (cat. no. MAB3050, Minneapolis, MN, USA) and Invitrogen (cat. no. A11001, Carlsbad, CA, USA) respectively. All other chemicals used in this study were of analytical grade.

2.2. Conjugation of the cRGDfK peptide with PEG-Lipid

The cRGDfK peptide was conjugated with NHS-PEG-DSPE (1.2:1 molar ratio) in deionized water at room temperature for 24 hrs. The conjugation of cRGDfK with PEG was confirmed by determining the molecular weight of cRGDfK-PEG-DSPE by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS (Bruker Daltonics, Germany) using acetonitril:water=7:3 with 0.1 % trifluoroacetate as the matrix solution, supplied with 10 mg/ml of dihydroxybenzoic acid.

2.3. Preparation of PEG-LPs for cellular uptake

PEGylated liposomes (PEG-LP) composed of EPC and Cholesterol (molar ratio: 7/3) were prepared by the lipid hydration method. Five mol% PEG-DSPE of the total lipid was added to the lipid solution. A lipid film was formed by evaporation of the solvents (chloroform and ethanol) from the lipid solution in a glass tube. HEPES buffer (10 mM, pH 7.4) was added, followed by a 10 min incubation to hydrate the lipid film. Finally, the glass tube was sonicated for approximately 30 sec in a bath-type sonicator (AU-25C, Aiwa, Tokyo, Japan). During film formation, 1 mol%
rhodamine-DOPE was incorporated to label the lipid composition. Five mol% cRGDFK-PEG-DSPe was added in place of PEG-DSPE in preparing RGD-PEG-LP and 2 mol% STR-R8 was also added to the lipid solution to produce the dual-ligand based PEG-LP (R8/RGD-PEG-LP).

2.4. Preparation of PEG-CLPs for transfection
In this experiment, a cationic lipid is used and, hence, the PEGylated liposomes are denoted as PEG-cationic liposomes (PEG-CLPs). PEG-CLP was prepared using DOTAP, DOPE and Cholesterol at a molar ratio of 3:4:3. Five mol% PEG-DSPE of the total lipid was added to the lipid solution. A lipid film was formed by evaporation of the solvents from the lipid solution in a glass tube. Luciferase-encoding pDNA (7037 bp) was condensed with protamine (N/P=1) to prepare the core in HEPES buffer. After condensation, pDNA solution (60 μg/ml) was added to the glass tube, followed by a 10 min incubation to hydrate the lipid film. Finally, the glass tube was sonicated for approximately 30 sec in a bath-type sonicator (AU-25C, Aiwa, Tokyo, Japan), followed by the same process as described above.

2.5. Characterization of PEG-LPs and PEG-CLPs
The mean size and zeta potential of the prepared PEG-LPs were determined using a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments Ltd., Worcestershire, UK).

2.6. Measurement of pDNA encapsulation efficiency
The encapsulation efficiency of plasmid DNA contained in the prepared PEG-CLPs was determined by following a previously described protocol [26]. Briefly, we used PicoGreen (Molecular Probes, Eugene, OR, USA) a membrane impermeable dye that specifically binds to double-stranded DNA and is fluorescent. Poly aspartic acid (100 μg/ml) was employed to maintain pDNA free from the condensed pDNA. PicoGreen fluorescence was measured using a Spectrofluorometer at excitation and emission wavelengths of 495 and 525 nm, respectively. Plasmid DNA encapsulation efficiency was determined from the fluorescence intensity upon the addition of PicoGreen to the PEG-CLPs and the values compared to those obtained upon lysis of the PEG-CLPs lipid bilayers with 10% Triton X-100.

2.7. Isolation of skin endothelial cells (Skin EC)
All procedures involving animals were performed in compliance with Hokkaido University guidelines, and the protocols were approved by the Institutional Animal Care and Use Committee. Skin endothelial cells (Skin EC) were isolated as previously described [27]. Briefly, the skin on the back of female mice was excised.
The excised tissue was minced and digested with collagenase II (Worthington, Freehold, NJ). Blood cells were removed by a single sucrose step-gradient centrifugation with Histopaque 1077 (Sigma-Aldrich), and the cell suspensions were filtered. Endothelial cells were isolated using MACS according to the manufacturer's instructions using a FITC-anti-CD31 antibody. CD31-positive cells were sorted and plated on 1.5% gelatin-coated culture plates and grown in EGM-2MV (Clonetics, Walkersville, MD) and 10% fetal calf serum. After subculturing for 2 weeks, the endothelial cells were purified by a second MACS using FITC-BS1-B4 to eliminate contaminating stromal cells and then cultured in EGM-2MV. All of the endothelial cells were split at a ratio of 1:3. The isolated ECs were purified by a second round of purification using FITC-BS1-B4.

2.8. Cell cultures
Human umbilical vein endothelial cells (HUVEC) were cultured in EBM-2 medium supplemented with 2% FBS (v/v), penicillin (100 units/ml), streptomycin (100 mg/ml) & bullet kits. Skin endothelial cells (Skin EC) were cultured in EBM-2 medium supplemented with 5% FBS (v/v), penicillin (100 units/ml), streptomycin (100 mg/ml) & bullet kits under an atmosphere of 5% CO₂ at 37 °C.

2.9. Evaluation of the expression of Integrin α₅β₃ receptor
Cells were washed with PBS (pH 7.4) and detached by treatment with trypsin-EDTA. Detached cells were incubated with anti Integrin α₅β₃ antibody for 20 min at 4 °C, followed by incubation with Alexa Flour 488 labeled secondary antibody for 20 min at 4 °C. Ten thousand cells per sample were analyzed using a FACSVantageSE flow cytometer (BD, San Jose, CA, USA).

2.10. Quantitative analysis of cellular uptake by Spectrofluorometer
For the cellular uptake study, 40,000 cells were seeded in a 24-well plate (Corning Incorporated, Corning, NY, USA) and incubated overnight at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. After 24 hrs, the prepared rhodamine labeled PEG-LPs were added and the suspension was incubated for 3 hrs at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. After 3 hrs, the cells were washed twice by adding 1ml of ice-cold phosphate buffer saline (PBS) supplemented with heparin (40 units/ml) [28] and were then treated with Reporter Lysis Buffer (Promega Corp., Madison, WI, USA) followed by centrifugation at 12,000 rpm for 5 min at 4 °C to remove debris. The cellular uptake efficiency of the prepared rhodamine labeled PEG-LPs were determined by measuring the fluorescence intensity of rhodamine (excitation at 555 nm and emission at 575 nm) using FP-750 Spectrofluorometer (JAS Co, Tokyo, Japan).
2.11. Qualitative analysis of cellular uptake by Confocal Laser Scanning Microscopy (CLSM)

To investigate the cellular uptake of the different PEG-LPs, 200,000 HUVEC cells were seeded on a 35-mm glass-bottom dish (Iwaki, Chiba, Japan) in 2 ml of culture medium for 24 h. The next day, the cells were washed once with 2 ml of PBS and then incubated with PEG-LPs (10nmol lipid/ml) in Kreb’s buffer for 3 h at 37 °C. After 2.5 h of incubation, 5 µl of Hoechst 33342 (1 mg/ml) was added to stain the nuclei and the suspension reincubated for an additional 30 min. The medium was then removed and the cells were washed twice by adding 1ml of PBS supplemented with heparin (40 units/ml). Finally, 1 ml of Krebs buffer was added and the cells were observed by confocal microscopy (A1 Confocal Laser Microscope System, Nikon Instruments Inc., Tokyo, Japan).

2.12. Evaluation of the cellular uptake mechanism by Spectrofluorometry

To investigate the mechanism of internalization of the modified PEG-LPs, 40,000 HUVEC 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. Before transfection, the cells were washed with 1 ml of PBS and then pre-incubated with Kreb’s buffer in the absence or presence of the following inhibitors for various times: Amiloride (5 mM) for 30 min; Sucrose (0.5M) for 30 min or Filipin III (40 µg/ml) for 1 h at 37 °C [28-30]. PEG-LPs were added and the cells were incubated for 1 h at 37 °C in the presence or absence of inhibitors. The cells were washed twice by adding 1 ml of PBS supplemented with heparin (40 units/ml) to completely remove the surface-bound PEG-LPs and the fluorescence intensity of rhodamine was then determined, as described above.

2.13. Transfection

Forty thousand cells were seeded in a 24-well plate, followed by incubation overnight at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. After 24 h, the cells were washed with PBS and condensed pDNA encapsulated PEG-LPs were added (0.8 µg of pDNA/well), followed by a 24 h reincubation. Luciferase gene expression was then evaluated based on photoluminescence intensity using a Luciferase assay kit (Luminescencer-PSN AB-2200, Atto Corporation, Tokyo, Japan). The amount of protein in each well was simultaneously determined using the BCA protein assay method.

2.14. Statistical analysis

Comparisons between multiple treatments were made using the one-way analysis of variance (ANOVA), followed by the Dunnett test. Pair-wise comparisons of subgroups were made using the student’s t-test. Differences among means were
considered to be statistically significant at a p value of <0.01.
3. Results

3.1. Conjugation of cRGDfK with PEG-lipid

cRGDfK-PEG-DSPE was synthesized after conjugation of the cRGDfK peptide (calculated MW 603.68 and observed MW 603.78) with NHS-PEG-DSPE (calculated Mn 2996) in PBS (Fig. 1A). A MALDI-TOF MS analysis was performed on the cRGDfK peptide and Maleimide-PEG-DSPE (Fig. 1B) and the conjugation of cRGDfK-PEG-DSPE was confirmed from fragment shifts (Fig. 1C). Free peptide present in the preparation was removed by dialysis against water.

![Conjugation Diagram](attachment:conjugation_diagram.png)

**Fig. 1. Conjugation of cRGDfK with NHS-PEG-DSPE.** (A) Synthesis of cRGDfK-PEG-DSPE. NHS-PEG-DSPE and the cRGDfK peptide (molar ratio 1:1.2) were dissolved in water at 37 °C and allowed to react for 24 h. MALDI-TOF MS spectra of (B) NHS-PEG-DSPE and (C) cRGDfK-PEG-DSPE.

3.2. Detection of Integrin $\alpha_v\beta_3$ receptor

To evaluate the expression of the Integrin $\alpha_v\beta_3$ receptor on the cell surface, flow cytometric analyses of HUVEC and Skin EC was carried out using Alexa Flour 488 labeled antibodies (Fig. 2). The flow cytometric analysis revealed that the HUVEC cells expressed substantial levels of Integrin $\alpha_v\beta_3$; but no expression of target receptor could be detected from the Skin EC. The expression of the Integrin
β3 subunit from Skin EC was also evaluated via the use of other types of IgG (anti-mouse Integrin β3 antibody, cat. no. sc-19671, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) but, in all cases, no expression of Integrin β3 was detected from mouse Skin EC (data not shown).

Fig. 2. Expression level of Integrin αvβ3
The expression of Integrin αvβ3 on (A) HUVEC, (B) Skin EC was confirmed by flow cytometry analysis as described in materials and methods. Black lines indicate non-treatment, and green lines indicate results obtained for the anti Integrin αvβ3 antibody treatment.

3.3. Quantitative evaluation of the cellular uptake of PEG-LP modified with dual-ligand
For the development of the dual-ligand system as well as for subsequent evaluation, we incorporated STR-R8 on the surface of the lipid membranes of PEG-LPs. The average diameter and zeta-potential of the modified PEG-LPs were then determined (Table 1). The findings showed that cRGDfK had no effect on the surface charge of PEG-LPs. The zeta-potential of LP was found to be nearly neutral (-0.438) but after adding 5 mol% PEG-DSPE, the zeta potential became negative. Therefore, it appears that the negative charge of PEG-LPs is mainly derived from phosphate groups those are present in DSPE. The surface charge of the PEG-LPs became positive after the incorporation of STR-R8.
Table 1 Physical properties of PEG-LPs prepared for cellular uptake study

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<td></td>
<td>Size (dnm)</td>
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<tr>
<td>PEG-LP</td>
<td>99±11</td>
</tr>
<tr>
<td>RGD-PEG-LP</td>
<td>93±7</td>
</tr>
<tr>
<td>R8/PEG-LP</td>
<td>97±19</td>
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<tr>
<td>R8/RGD-PEG-LP</td>
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The data are expressed as the mean ± SD value from at least three different preparations.

The relative comparison of the extent of cellular uptake for the single and or dual-ligand systems using different cell lines is shown in Fig. 3, where the uptake of PEG-LP modified with 5 mol% of PEG was set as control. In HUVEC, about a 1.4 fold enhancement in cellular uptake was observed for the 5 mol% RGD-PEG modified single-ligand version (RGD-PEG-LP) as compared to the control, indicating that the RGD motif grafted to the terminal ends of PEG molecules might recognize target receptors. After the addition of 2 mol% STR-R8 to the surface of the lipid membrane of 5 mol% PEG modified LP (R8/PEG-LP), cellular uptake was increased by 2.2 fold (vs. PEG-LP). However, in the presence of STR-R8, a significant difference in cellular uptake was observed between R8/PEG-LP and R8/RGD-PEG-LP (Fig. 3A). Compared with the control, the STR-R8 modified dual-ligand system (R8/RGD-PEG-LP) showed about a 5.1 fold increment in cellular uptake, indicating that, in presence of STR-R8, the RGD motif functions efficiently and exerts a significant enhancement in cellular uptake. This enhancement in cellular uptake is the result of specific interactions of the RGD motif with targeted Integrin αvβ3 expressed from HUVEC, along with the presence of STR-R8, which allows the well-organized internalization of PEG-LP on the targeted cell surface. To the contrary, in Skin EC, no significant difference in cellular uptake was observed between PEG-LP and RGD-PEG-LP. In contrast to PEG-LP, STR-R8 modified PEG-LP (R8/PEG-LP) exhibited about a 5.8 fold enhancement in cellular uptake (Fig. 3B) which can be attributed to electrostatic interactions of R8 with the anionic cell surface. However, no significant difference in cellular uptake was observed for R8/PEG-LP and R8/RGD-PEG-LP, indicating the absence of specific interactions between the RGD motif and target receptors on the cell surface. It therefore appears that, in the case of Skin EC, the enhancement in cellular uptake in R8/PEG-LP and R8/RGD-PEG-LP was derived
only from the R8 peptide. The incorporation of STR-R8 as a CPP ligand in the dual-ligand system exhibited a synergistic effect on the cellular uptake of PEG-LP in Integrin αvβ3 expressing cells.

Fig. 3. Cellular uptake of Dual-ligand PEGylated liposomes
Different formulations of PEG-LPs were incubated with (A) HUVEC, (B) Skin EC for 3 h and the amount of cellular uptake was then determined, as described in material and methods section. Cellular uptake is expressed as the mean ± SD (n=3). The statistical differences v.s. PEG-LP were determined by one-way ANOVA followed by Dunnett test, *P<0.05, **P<0.01. Comparisons between R8/PEG-LP and R8/RGD-PEG-LP were determined by Student’s t-test. **P < 0.01, not significant (N.S.)

3.4. Qualitative evaluation of the cellular uptake of PEG-LP modified with dual-ligand
The cellular uptake of PEG-LPs by HUVEC was investigated by CLSM, as shown in Fig. 4. The cellular uptake of PEG-LP was set as a control. The CLSM study showed that a very weak fluorescence signal was obtained for PEG-LP, indicating that only small amounts of PEG-LP were internalized into the cells (Fig.4A). Compared to the control, the RGD modified single ligand system (RGD-PEG-LP) resulted in a higher amount of internalization (Fig.4B), indicating that the RGD motif has the ability to recognize targeted Integrin αvβ3 receptors expressed on the surface of the cells. STR-R8 modified PEG-LP (R8/PEG-LP) was not internalized efficiently and its level of internalization was similar to that for PEG-LP (Fig.4C).
However, the use of STR-R8 modified RGD-PEG-LP (R8/RGD-PEG-LP) resulted in strong fluorescence signals inside the cells (Fig.4D), indicating that R8/RGD-PEG-LP was efficiently internalized by the cells and this result is consistent with the findings of the fluorescence intensity study, as shown in Fig.3.

3.5. Investigation of the mechanism involved in the uptake of Dual-ligand PEGylated liposomes
The cellular uptake pathway for the modified PEG-LPs was examined by evaluating the contribution of the different endocytic pathways. Inhibitors that specifically block macropinocytosis, clathrin-mediated endocytosis and
Caveolae-mediated endocytosis were used to determine the uptake mechanism of PEG-LPs. Specifically, a hypertonic medium (sucrose) was used to inhibit clathrin-mediated endocytosis via dissociation of the clathrin lattice; amiloride inhibits macropinocytosis by inhibiting the Na\(^+\)/H\(^+\) exchange required for macropinocytosis and filipin inhibits caveolar uptake through cholesterol depletion [28-30]. After performing the quantitative analyses it was found that the cellular uptake of RGD-PEG-LP was largely inhibited by filipin and partially by sucrose (Fig. 5), indicating that RGD modified PEG-LP is mainly taken up by the caveolae-mediated endocytosis pathway. On the other hand, the cellular uptake of R8/PEG-LP was inhibited in the presence of sucrose, indicating that STR-R8 modified PEG-LP is taken up by the clathrin-mediated endocytosis pathway. However, the internalization of R8 and RGD modified PEG-LP (R8/RGD-PEG-LP) was significantly inhibited by sucrose, demonstrating that the dual-ligand modified PEG-LP is mainly taken up by the clathrin-mediated endocytosis pathway.

**Fig. 5. Percent inhibition of cellular uptake of PEG-LPs in the presence of specific inhibitors.**

HUVEC cells were incubated with rhodamine labeled PEG-LPs for 1 h in the presence of amiloride, sucrose and filipin. Black bar indicates the percentage of inhibition of cellular uptake by sucrose and the hatched bar by filipin. The relative cellular uptake was expressed as percentage of fluorescence measured in presence of inhibitors in each formulation. (No inhibition of cellular uptake was observed in presence of amiloride: hence, it is not shown in this figure)
3.6. Evaluation of gene expression

For the gene expression study, different formulations of PEG-CLPs were prepared by encapsulating the condensed pDNA core and the average diameter and zeta-potential of the modified PEG-CLPs were determined (Table 2). The observed surface charge indicated that the condensed pDNA was successfully encapsulated in the modified PEG-CLPs. In addition, the surface charges of empty PEG-CLP, RGD-PEG-CLP, R8/PEG-CLP and R8/RGD-PEG-CLP were determined and the value were found to be 28±4, 27±6, 36±5 and 32±6 mV respectively, indicating that there is no major difference in surface charge in between empty PEG-CLPs and pDNA encapsulated PEG-CLPs. The encapsulation efficiencies of pDNA in PEG-CLP, RGD-PEG-CLP, R8/PEG-CLP and R8/RGD-PEG-CLP were 70%, 73%, 78% and 80% respectively.

Table 2 Physical properties of PEG-CLPs prepared for transfection study

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<td>pDNA core</td>
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<td>R8/RGD-PEG-CLP</td>
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The data are expressed as the mean ± SD value from three different preparations.

Pharmacological applications of the dual-ligand system were evaluated by examining luciferase gene expression efficiency in HUVEC and in Skin EC and the results are shown in Fig.6. Condensed pDNA encapsulated PEG-CLP was used as the control. Compared with the control, the gene expression for RGD modified PEG-CLP (RGD-PEG-CLP) was about 6.5 fold higher (Fig. 6A). About a 3.5 magnitude increase in gene expression vs PEG-CLP was found for STR-R8 modified PEG-CLP (R8/PEG-CLP). However, STR-R8 modified RGD-PEG-CLP (R8/RGD-PEG-CLP) showed a significantly higher level of gene expression than the other PEG-CLPs and the level was about 300 fold higher compared to PEG-CLP. It is reasonable to assume that RGD motif peptide grafted to the terminal end of the PEG chain of PEG-CLP might efficiently recognize Integrin αvβ3 expressed on surface of HUVEC. On the other hand, in Skin EC, only a 1.4 fold higher gene expression was observed for RGD-PEG-CLP compared to PEG-CLP (Fig. 6B). STR-R8 modified RGD-PEG-CLP (R8/RGD-PEG-CLP)
showed about a 10 times higher level of gene expression. However, no significant difference in gene expression was observed from modified PEG-CLPs as compared with the control.

Fig. 6. Effect of Dual-ligand PEGylated cationic liposomes (PEG-CLPs) on gene expression
Different formulations of PEG-CLPs encapsulated with pDNA were incubated with (A) HUVEC, (B) Skin EC for 24 h and the level of gene expression was then determined as described in the material and methods section. The results are expressed as the mean ± SD (n=3). Comparisons between R8/RGD-PEG-CLP vs others were determined by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. **P < 0.01, not significant (N.S.)
4. Discussion

Nontargeted PEG-LPs (as stealth liposomes), e.g., Doxil, have been extensively used for delivering cytotoxic drugs to tumors via the EPR effect [31]. The active targeting of these particles resulted in further improvement in antitumor activity, therapeutic efficacy with less toxicity. Due to the PEG dilemma, the active targeting of PEG-LPs fails to permit efficient association with the target cells. As a result, the delivery device cannot reach the target sites and this ultimately leads to the loss of pharmacological applications of PEG-LPs [3]. To overcome the PEG dilemma, the design of an effective active targeting PEGylated liposomal delivery system that not only enhances the cellular internalization of the particles but also exerts better therapeutic effects, is highly desirable.

In this study, Inteigrin αvβ3 was employed as the target receptor, because it is highly expressed by tumor cells as well as tumor endothelial cells [32-34]. It has been reported that the RGD motif peptide, either in linear or cyclic form, recognizes Inteigrin αvβ3 and that it can be attached to the PEG-LPs or polyplex micelles for the successful delivery of drugs to target sites [35-38]. In this study, we attached the RGD motif peptide (cRGDfK) to the terminal ends of PEG molecules of PEG-LP as a single ligand system (RGD-PEG-LP) and STR-R8 was then attached to the surface of the lipid membrane of RGD-PEG-LP to prepare a dual-ligand system (R8/RGD-PEG-LP), as shown in Fig.7. The RGD containing single-ligand system showed minor or negligible effects for the cellular uptake of PEG-LP, even in Integrin αvβ3 expressing cells, as shown in Fig. 3. These results support the assumption that a specific ligand would have the capability to internalize the PEG-LP on the target cell surface but cannot exert significant effects on the cellular uptake of PEG-LPs and, in this regard, steric hindrance by PEG might be responsible for the suppression of cellular uptake. To ensure better cell selectivity or therapeutic efficacy, a dual-targeting system mediated by two types of specific ligands was used in previous studies [39-40]. The dual-targeting system had the ability to confer more efficient pharmacological effects than that of a single ligand version. However the uptake pathway mediated by the specific ligand is mainly receptor endocytosis which is saturated and limits the amount of cellular uptake of liposomes. To enhance the cellular uptake mediated by a specific ligand, CPP has also been used in a dual-ligand system [41].
Although the property of CPPs is highly promising for in vitro delivery, they have a severe disadvantage for in vivo systemic applications, because of non-specific interactions of their cationic charge with biological components, resulting in inactivated or collapsed liposomal formulations. Therefore, the development of an advanced design that permits the property of CPPs to be manipulated with respect to the in vivo situation would result in a more valuable and useful active targeting system.

In this study we propose the use of a dual-ligand system mediated by a specific ligand and CPP to achieve cell selectivity, efficient cellular uptake as well as to observe the pharmacological effects of PEG-LP. For this purpose, STR-R8 as the CPP was attached to PEG-LP on the surface of the lipid membrane. Based on the results shown in Fig. 3, the dual-ligand system modified with the RGD motif peptide and STR-R8 (R8/RGD-PEG-LP) showed synergistic effects on the cellular uptake in HUVEC. The RGD motif peptide modified PEG-LP increased cellular uptake by 1.4 fold indicating that a single ligand could recognize the target receptor but might not be efficient enough to overcome the steric hindrance of PEG to exert the proper internalization of PEG-LP. Although STR-R8 modified
PEG-LP (R8/PEG-LP) showed a slight increase in cellular uptake, probably due to the cationic behavior of the R8 peptide as the CPP ligand, a further enhancement in cellular uptake was observed when STR-R8 was modified with RGD-PEG-LP (R8/RGD-PEG-LP). This indicates that, after recognition of the target receptor by the RGD motif, the R8 peptide participates in electrostatic interactions with the cell surface that ultimately permit the PEG-LP to overcome the steric hindrance of PEG. These results suggest that the dual-ligand system shows a synergistic effect and would be capable of enhancing the cellular uptake of PEG-LP. However, in Skin EC, the RGD modified PEG-LP did not enhance cellular uptake, compared to PEG-LP. STR-R8 modified PEG-LP (R8/PEG-LP) enhanced cellular uptake in a greater extent. In contrast to R8/PEG-LP, R8 modified RGD-PEG-LP (R8/RGD-PEG-LP) failed to further enhance cellular uptake which is due to lack of appropriate interactions of the RGD motif with the targeted receptor on the cell surface. The enhancement in cellular uptake mediated by the dual ligand modified PEG-LP (R8/RGD-PEG-LP) in HUVEC was also verified in a CLSM study, as shown in Fig. 4. This study showed that PEG-LP is not extensively internalized into the cells and this can be attributed to effects of PEG on the cellular association of the particles. RGD modification on the distal part of PEG of PEG-LP (RGD-PEG-LP) increases the fluorescence signal mediated by the interaction of the RGD motif peptide with the target Integrin αvβ3 receptor. This study also confirms that STR-R8 modification on the surface of lipid membrane of PEG-LP (R8/PEG-LP) is not efficient in terms of internalizing PEG-LP, although the fluorescence intensity study of R8/PEG-LP indicated about a 2.2 fold increment in cellular uptake as compared with PEG-LP (Fig.3). However, the simultaneous modification of PEG-LP with the RGD motif and STR-R8 (R8/RGD-PEG-LP) results in a product that gave a strong fluorescence signal, indicating that the dual ligand modified PEG-LP is efficiently internalized into the target cells. The CLSM analysis also confirmed a significant enhancement in cellular uptake, as mediated by the dual-ligand present in PEG-LP (R8/RGD-PEG-LP) (Fig.4). These results suggest that the dual-ligand system would be an effective design for a PEG-LP that would show better cellular association as well as accelerate cellular uptake. To determine the cellular uptake route of the modified PEG-LPs (Fig. 5), the findings show that the STR-R8 modified PEG-LP (R8/PEG-LP) is taken up by the clathrin-mediated endocytosis pathway. Generally, LPs modified with lower concentrations of R8 are taken up by clathrin-mediated endocytosis, but LPs modified with higher concentrations of R8 follow macropinocytosis as the uptake route [42]. However this phenomenon depends on the cell lines in use. A recent study reported that the cellular uptake
pathway of R8 modified LP also depends on the position of R8 i.e. whether it is on the surface of the lipid membrane of LP or on the top of the PEG [43]. The RGD modified PEG-LP (RGD-PEG-LP) is taken up mainly by caveolae-mediated endocytosis, which is consistent with findings reported by other research groups [36, 44]. Interestingly, it was observed that, after the incorporation of R8 and RGD into the PEG-LP, the internalization of R8/RGD-PEG-LP is predominantly governed by the clathrin-mediated endocytosis pathway. These results indicate that after the identification of Integrin $\alpha_v\beta_3$ receptors by the RGD motif, the PEG-LP comes into close contact with the cell surface which allows R8 to participate in electrostatic interactions with the cell surface and finally the cellular uptake of dual-ligand PEG-LP is presided over by R8 which might play a significant role in the observed synergistic effect of R8/RGD-PEG-LP. This effect serves to confirm our hypothetical design of a dual-ligand system, as shown in Fig. 7. A gene expression study was performed to observe the pharmacological activity of the designed dual-ligand system and the findings are shown in Fig. 6. The findings show that condensed pDNA encapsulated PEG-CLPs modified with either the RGD motif peptide or STR-R8 are not sufficient to achieve gene delivery, whereas, the RGD motif peptide and STR-R8 modified PEG-CLP (R8/RGD-PEG-CLP) shows better transfection efficiency as well as successfully delivered higher amounts of gene products in Integrin $\alpha_v\beta_3$ expressing cells. Therefore, this newly-developed dual-ligand system has the possibility of providing better cellular association of PEGylated liposomes even in in vivo conditions and consequently would be an effective design for the delivery of chemotherapeutics or any macromolecules in cancer treatment in the future.
5. Conclusion
We report herein on the successful design of a dual-ligand system comprised of a specific ligand and a CPP ligand for the targeted delivery of PEG-LP to Integrin $\alpha_v\beta_3$ expressing cells. Based on the design of the dual-ligand system, STR-R8 shows activity as a CPP ligand. The cellular uptake of RGD modified PEG-LP follows the caveolae-mediated endocytosis pathway, but, in the case of the dual-ligand system containing the RGD motif in association with STR-R8, the uptake route is preferentially governed by the clathrin-mediated endocytosis pathway. A single ligand system with a RGD motif shows a negligible or almost equal amount of cellular uptake compared to PEG-LP, whereas the dual-ligand system functions synergistically to enhance cellular uptake as well as to play a significant role in gene delivery to the target cells. Our proposed protocol indicates the scope for future in vivo applications in tumor bearing animal bodies.
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References


Table Captions

Table 1 Physical properties of PEG-LPs prepared for cellular uptake study
The data are expressed as the mean ± SD value from at least three different preparations.

Table 2 Physical properties of PEG-CLPs prepared for transfection study
The data are expressed as the mean ± SD value from three different preparations.
Figure Captions

Fig. 1. Conjugation of cRGDfK with NHS-PEG-DSPE. (A) Synthesis of cRGDfK-PEG-DSPE. NHS-PEG-DSPE and the cRGDfK peptide (molar ratio 1:1.2) were dissolved in water at 37 °C and allowed to react for 24 h. MALDI-TOF MS spectra of (B) NHS-PEG-DSPE and (C) cRGDfK-PEG-DSPE.

Fig. 2. Expression level of Integrin αvβ3
The expression of Integrin αvβ3 on (A) HUVEC, (B) Skin EC was confirmed by flow cytometry analysis as described in materials and methods. Black lines indicate non-treatment, and green lines indicate results obtained for the anti Integrin αvβ3 antibody treatment.

Fig. 3. Cellular uptake of Dual-ligand PEGylated liposomes
Different formulations of PEG-LPs were incubated with (A) HUVEC, (B) Skin EC for 3 h and the amount of cellular uptake was then determined, as described in material and methods section. Cellular uptake is expressed as the mean ± SD (n=3). The statistical differences v.s. PEG-LP were determined by one-way ANOVA followed by Dunnett test, *P<0.05, **P<0.01. Comparisons between R8/PEG-LP and R8/RGD-PEG-LP were determined by Student’s t-test. **P < 0.01, not significant (N.S.)

Fig. 4. Uptake of Dual-ligand PEGylated liposomes in HUVEC observed by confocal laser scanning microscopy (CLSM).
Representative confocal microscopic images of HUVEC incubated with (A) PEG-LP, (B) RGD-PEG-LP, (C) R8/PEG-LP, (D) R8/RGD-PEG-LP containing rhodamine lipid phase for 3 h. The nucleus was stained with Hoechst 33342. The scale bars represent 25 μm.
Fig. 5. Percent inhibition of cellular uptake of PEG-LPs in the presence of specific inhibitors.
HUVEC cells were incubated with rhodamine labeled PEG-LPs for 1 h in the presence of amiloride, sucrose and filipin. Black bar indicates the percentage of inhibition of cellular uptake by sucrose and the hatched bar by filipin. The relative cellular uptake was expressed as percentage of fluorescence measured in presence of inhibitors in each formulation. (No inhibition of cellular uptake was observed in presence of amiloride: hence, it is not shown in this figure)

Fig. 6. Effect of Dual-ligand PEGylated cationic liposomes (PEG-CLPs) on gene expression
Different formulations of PEG-CLPs encapsulated with pDNA were incubated with (A) HUVEC, (B) Skin EC for 24 h and the level of gene expression was then determined as described in the material and methods section. The results are expressed as the mean ± SD (n=3). Comparisons between R8/RGD-PEG-CLP vs others were determined by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. **P < 0.01, not significant (N.S.)

Fig. 7. Schematic representation of single ligand and dual-ligand modified PEGylated liposomes.