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**Title:**

Design of a dual-ligand system using a specific ligand and cell penetrating peptide, resulting in a synergistic effect on selectivity and cellular uptake

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## **Abstract**

In this study, a dual-ligand liposomal system comprised of a specific ligand and a cell penetrating peptide (CPP) is described to enhance selectivity and cellular uptake. Dual-ligand PEGylated liposomes were prepared by modifying the end of the PEG with an NGR motif peptide, followed by a surface coating of the liposomes with stearylated oligoarginine (STR-RX). The NGR motif recognizes CD13, a marker protein located on tumor endothelial cells. A suitable number of RX units was determined to be R4, since it can be masked by the PEG aqueous layer. Although no enhanced cellular uptake was observed when a single modification of PEGylated liposomes with either NGR- or STR-R4 was used, the dual-modification with NGR and STR-R4 stimulated uptake of PEGylated liposomes by CD13 positive cells, and this uptake was superior to that obtained by PEG-unmodified liposomes modified with STR-R4. The dual-ligand system shows a synergistic effect on cellular uptake. Collectively, the dual-ligand system promises to be useful in the development efficient and specific drug delivery systems.

## **Key words**

dual-ligand, active targeting, cell penetrating peptide, NGR, tumor endothelial cells

## 1. Introduction

The use of liposomes in drug delivery systems (DDS) is emerging as a viable class of therapeutics for use in the treatment of human diseases (Torchilin, 2005). Long-circulating liposomes, produced by modification with poly(ethylene glycol) (PEG) are able to passively accumulate in tumors via the enhanced permeability and retention (EPR) effect. To achieve further chemotherapeutic efficacy, tumor targeting PEGylated liposomes have been developed by attaching specific targeting ligands to the distal end of the PEG chain (Suzuki et al., 2008; Yamada et al., 2008; Fondell et al., 2010; Elbayoumi et al., 2010; Hatakeyama et al., 2007a). In general, specific interactions between ligands and target molecules induce the receptor mediated endocytosis of liposomes (Khalil et al., 2006a). Although it is possible to achieve active targeting via the use of specific ligands, receptor mediated endocytosis is a saturated pathway, which restricts the amount of liposomes that are available for cellular uptake. In addition, following cellular uptake, the endosomes fuse with lysosomes for degradation. Thus, the introduction of specific ligands onto PEGylated liposomes cannot be considered to be a complete solution for achieving the enhanced therapeutic activity.

On the other hand, cell-penetrating peptides (CPPs) are widely utilized for the delivery of drugs and genes (El-Sayed et al., 2009). CPPs, also known as protein transduction domains (PTDs) are capable of entering cells efficiently, either alone or linked to, not only small molecules, but also bulky cargos such as peptides, proteins, oligonucleotides, plasmid DNA

(pDNA), and liposomes. In the previous report, we described the development of stearylated octaarginine (STR-R8)-modified liposomal nanoparticles which are efficiently internalized into cells. The use of such products results a substantial increase in the intracellular transport of their cargos, which can include small molecules, pDNA, oligodeoxynucleotide (ODN), small interfering RNA (siRNA), and proteins in culture cells (Kogure et al., 2004; Nakamura et al., 2006; Nakamura et al.,2007; Suzuki et al.,2007). Despite the ability of CPPs to efficiently carry molecules into cells, it is not possible to apply CPPs-modified liposomes to in vivo systemic administration, due to of their non-specificity in cellular association and biodistribution.

Taking both specific ligands-mediated active targeting and CCPs-mediated efficient intracellular delivery into consideration, a rational strategy designed to take advantage of a combination of both specific ligands and CPPs could allow PEGylated liposomes to be used as a more selective and efficient in vivo systemic application. In the present study, we described the development of a dual-ligand system, in which specific ligand-modified PEGylated liposomes are combined with CPPs, which function as cationic ligands. In order to develop an ideal dual-ligand system, it is important to manipulate each ligand so that its synergistic function is exerted at the correct time and location as follows: CPPs should be unfunctional and opsonin-free in the systemic circulation due to steric hindrance of the PEG layer. After their arrival at the target site, cellular association via specific ligands permits CPPs to exert

their powerful ability to internalize the liposomes into target cells due to the close proximity of the liposomes to the surface of the target cells.

To address these issues, we examined the basic design and utility of a dual-ligand system in detail. The number of arginine residues was altered, in an attempt to find a suitable form of stearylated oligoarginine (STR-RX) for use in a dual-ligand system. CD13, a marker of angiogenic endothelial cells, which is recognized by the NGR peptide motif, was employed as a target molecule (Pastorino et al., 2003). The extent of cellular uptake of dual-ligand formulations were compared with that of single-ligand versions using not only model cells but also primary cultured tumor endothelial cells that has been isolated from tumor xenografts.

## 2. Materials and Methods

### 2.1. Materials

Cholesterol (Chol), distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*′[methoxy(polyethylene glycol)-2000] (PEG-DSPE), rhodamine-labeled DOPE (Rho-DOPE) were purchased from AVANTI Polar Lipids (Alabaster, AL, U.S.A.). PEG-DSPE with a functional maleimide moiety at the terminal end of PEG: *N*′[(3-maleimide-1-oxopropyl) aminopropyl polyethyleneglycol-carbamyl] distearoylphosphatidyl-ethanolamine (Mal-PEG-DSPE) and egg phosphatidylcholine (EPC) were purchased from Nippon Oil and Fat Co. (Tokyo, Japan). Stearylated octaarginine (STR-R8), hexaarginine (STR-R6), tetraarginine (STR-R4), and diarginine (STR-R2) was purchased from PolyPeptide Laboratories (San Diego, CA, U.S.A.). NGR motif peptide, CYGGRGNG was purchased from Sigma Genosys Japan (Ishikari, Japan). Rat Anti Mouse CD13 antibody was purchased from AbD Serotec (Oxford, U.K.). Alexa Fluor 488 labeled goat anti-rat IgG was purchased from Invitrogen (Carlsbad, CA, U.S.A.). FITC labeled anti-CD31 was obtained by eBioscience (San Diego, CA, U.S.A.). FITC-BS1-B4 was purchased from by Vector Laboratories (Burlingame, CA, U.S.A.). All other chemicals used were commercially available reagent-grade products.

### 2.2. Synthesis of NGR peptide conjugated PEG-lipid (NGR-PEG-lipid)

Equimolar quantities of NGR motif peptide and Mal-PEG-DSPE were mixed in water at

room temperature for 24 hr. Conjugation of the peptide to Mal-PEG-DSPE was confirmed by determining the molecular weight of the resulting NGR-PEG-DSPE using MALDI-TOF MS (obsd.  $M_n=3719.64$ , calcd.  $M_n= 3720.82$ ).

### **2.3. Preparation of liposomes**

Liposomes were prepared from EPC, Chol and Rho-DOPE (molar ratio: 70:30:1). All lipids were dissolved in a chloroform/ethanol solution. After evaporation under nitrogen gas, the dried lipid film was hydrated in PBS. The lipid film in PBS was sonicated for approximately 30 sec in a bath-type sonicator (AU-25C, Aiwa, Tokyo, Japan), followed by sonication for approximately 10 min in a probe-type sonicator (S3000, Misonix, NY, U.S.A.). For modification of the prepared liposomes with STR-RX, PEG-DSPE, or NGR-PEG-DSPE, liposomes were incubated with the indicated amounts of STR-RX, PEG-DSPE, or NGR-PEG-DSPE for 60 min at 55°C, 950 rpm in a SHAKING INCUBATOR SI-300 (AS ONE, Osaka, Japan). The average diameter and the zeta-potential of the prepared liposomes were measured using a Zetasizer Nano ZS ZEN3600 (MALVERN Instrument, Worchestershire, U.K.).

### **2.4. Isolation of tumor endothelial (TE) cells**

SM-ECs, melanoma TE cells, were isolated as described previously (Ohga et al., 2009) In

brief, SM-ECs were isolated from xenografted melanoma tissues (A375SM) in nude mice aged 8–12 weeks (Sankyo Labo, Tokyo, Japan). SM-ECs were isolated using a magnetic cell sorting system (Miltenyi Biotec, Tokyo, Japan) according to the manufacturer's instructions using a FITC–anti-CD31 antibody. CD31-positive cells were sorted and plated onto 1.5% gelatin-coated culture plates and grown in EGM-2 MV (Clonetics, Walkersville, MD, U.S.A.) and 15% FBS. Diphtheria toxin (500 ng/mL; Calbiochem, San Diego, CA, U.S.A.) was added to SM-EC subcultures to kill any remaining human tumor cells. The isolated SM-ECs were purified by a second round of purification, using the FITC–BS1-B4 antibody, and purity was determined by flow cytometry. Peripheral blood-derived endothelial cells (EPCs) were isolated and grown as previously described with minor modifications (Beaidry et al., 2007). Briefly, mouse peripheral blood was obtained by cardiocentesis. Peripheral blood mononuclear cells (PBMCs) were isolated using a Histopaque 1077 (Sigma Chemical Co., St. Louis, MO, U.S.A.) and centrifuged as per the manufacturer's instructions. The intermediate phase was collected and washed with HBSS before resuspension in EGM-2 MV supplemented with 15% FBS. PBMCs ( $1 \times 10^6$  per well) were seeded onto 12-well tissue culture plates coated with 0.5% gelatin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and a 2  $\mu$ g/mL solution of human fibronectin (Chemicon International, Temecula, CA, USA). Cells were passaged at a 1:3 ratio until they reached 90% confluence. PBMCs were regarded as EPCs, since they were Sca-1-positive in culture. All animal procedures were carried out in compliance with the

Hokkaido University guidelines, and the protocols were approved by the Institutional Animal Care and Use Committee.

## **2.5. Cell cultures**

MS-1 cells were cultured in DMEM (SIGMA-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% humidity. TE cells were cultured in BulletKit (LONZA, Basel, Switzerland) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% humidity.

## **2.6. Determination of CD13 expression**

Cells were detached by treatment with trypsin-EDTA and incubated with anti CD13 antibodies, followed by incubation with secondary antibodies labeled with Alexa 488 for 20 min at 4 °C. Ten thousand cells per sample were analyzed using a FACSVantageSE flow cytometer (BD, San Jose, CA, U.S.A.).

## **2.7. Evaluation of cellular uptake of liposomes**

MS-1 and TE cells were seeded on 24-well plates (40,000 cells per well) 1 day prior to evaluation. The prepared liposomes labeled with Rho-DOPE, were added to 500 µl of

Krebs-Henseleit buffer (0.12 mM of final lipid concentration) and were incubated with cells for 3 hr at 37°C. After washing the cells with Krebs-Henseleit buffer, they were lysed with Reporter Lysis Buffer (Promega, Madison, WI, U.S.A), followed by centrifugation at 12,000 rpm, for 5 min, at 4 °C to remove debris. Fluorescence intensity of rhodamine in the supernatant was determined using FP-750 Spectrofluorometer (JAS Co, Tokyo, Japan) (Excitation: 555 nm, Emission: 575 nm).

## **2.8. Statistic analysis**

Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the Dunnett test. Pair-wise comparisons between treatments were made using a student's t-test. A P-value of < 0.05 was considered to be significant.

### 3. Results

#### 3.1. Optimization of the number of arginine residue

To develop the proposed dual-ligand system, it should be noted that CPP ligands are not functional in the presence of PEG. To identify a suitable CPP ligand for the dual-ligand system, we first evaluated the effect of the number of arginine residues contained in STR-RX on the cellular association of PEGylated liposomes (PEG-LPs). The concentrations of PEG-lipid and STR-RX were set at 5 mol% of the total lipid. The physical properties of the prepared formulations are listed in Table 1. The diameters of the liposomes (LPs) and PEG-LPs with or without STR-RX were similar. However, the zeta-potentials were increased, depending on the number of arginine residues, and PEGylation reduced the zeta-potentials due to the partial or complete masking of STR-RX on the surface by the aqueous layer of the PEG moiety. Cellular uptake of the prepared formulations was evaluated using TE cells as shown in Fig. 1. The amount of uptake was correlated with their zeta-potentials. Even though the modification of LP with STR-R2 resulted in a positive charged particle, cellular uptake was comparable to that of an arginine-unmodified LP. On the other hand, the use of other types of STR-RX (STR-R4, R6, and R8) resulted in an enhancement of the cellular uptake and this uptake was dependent on the number of arginine residues. Despite the presence of PEG, the STR-R6 and R8-modified PEG-LPs were both taken up efficiently by cells. However, the uptake of PEG-LP prepared using STR-R4 was significantly inhibited down to the amount of

arginine unmodified LP. These results indicate that STR-R4 is likely masked by the PEG layer and represents acceptable CPP for use in the dual-ligand system.

### **3.2. Cellular uptake of PEGylated liposomes modified with single NGR ligand**

We next evaluated the single ligand-mediated cellular uptake of NGR modified PEG-LPs. The expression of CD13 on MS-1 and TE cells was confirmed by FACS analysis, as shown in Fig.2. Liposomes were modified with PEG-lipid or NGR-PEG-lipid at 1, 2, 5, 10, or 15 mol% of the total lipid. The PEG-LPs and NGR-PEG-LPs showed almost identical physical properties at every concentration of PEG-lipid, as shown in Table. 2. The amount of uptake of PEG-LPs was decreased with increasing the concentrations of PEG-lipid. Similar to PEG-LPs, NGR-PEG-LPs were not effective in either of the cell lines (Fig.3). Therefore, no enhancement in cellular uptake was observed in the case of the single ligand-mediated system.

### **3.3. The evaluation of dual-ligand system on the cellular uptake**

To develop a dual-ligand system, we combined the NGR-PEG-LPs with STR-R4. Since the ability of CPPs to associate with cells is quite intense and non-specific, due to electrostatic interactions with negative molecules on the cell surface, conditions should be considered where specific ligands exert their selectivity in the presence of CPPs. Therefore, we explored suitable conditions that would permit STR-R4 to express the selectivity of the NGR ligand. As

a result, the optimal concentration of STR-R4 on the NGR-PEG-LPs was determined to be 0.25 mol% of the total lipid. In this condition, the average diameters and zeta-potentials of NGR-PEG-LPs with STR-R4 (NGR-PEG/R4-LPs) remained unchanged, compared to NGR-PEG-LPs, as shown in Table 2. The cellular uptake of STR-R4 modified liposome (R4-LP) by MS-1 cells was 1.7-fold higher than that of unmodified liposome (LP) (Fig.4a gray bars), and the amount of PEG/R4-LP taken up was gradually decreased, depending on the concentration of PEG-lipid (Fig.4a open bars). These results indicate that PEGylation has a negative effect on cellular uptake. Comparisons of the relative amount of uptake are also summarized in Table 3, in which the uptake of LP is used as the control. On the other hand, the cellular uptake of the dual-ligand formulations (NGR-PEG/R4-LPs) was increased by 2.7-fold, when the liposome was modified with 10 mol% NGR-PEG-lipid compared with LP (Fig 4a gray bar vs. black bars). The addition of a higher amount of PEG at 15 mol% resulted in a slight decrease in cellular uptake, probably because of the steric hinderance of PEG. Unlike the single-ligand formulations, the NGR ligand in the dual formulations facilitated cellular uptake when PEG-lipid was present in the range of 5 to 15 mol% of total lipid even though PEG exerted a negative effect on cellular uptake. These improvements can be attributed to specific interactions between NGR and the target CD13, and the resulting close proximity of R4 to the cell surface, which permits R4 to internalize the liposomes efficiently into the target cells. As shown in Fig. 4b, a synergistic effect of the dual-ligand system on

cellular uptake was also observed in TE cells with lower concentrations of NGR-PEG-lipid than were used in MS-1 cells, presumably because the optimal conditions for expressing the dual-ligand effect is different for each type of cells. The modification of STR-R8 as a CPP ligand in dual-ligand formulations (NGR-PEG/R8-LPs) also resulted in a synergistic effect on cellular uptake (Supplementary Fig. 1 and Table 1). These results suggest that a dual-ligand design has an ability of the PEGylated liposomes to further enhance the selectivity and the amount of cellular uptake.

#### 4. Discussion

Although the delivery of chemotherapeutics for cancer can be achieved via the use of PEGylated liposomes based on the EPR effect, PEGylation reduces the extent of interactions of liposomes with target tumor cells, resulting in a reduced cellular uptake and poor endosomal escape due to steric hindrance. Overall, this results in a significant loss in the pharmacological effect of the cargos. The crucial negative effect of PEGylation on activity at a target site is referred to as the “PEG dilemma”, which hampers further practical applications of liposomal drugs and macromolecules (Hatakeyama et al., 2007b). To overcome this, specific ligands such as transferrin, folic acids, peptides, or antibodies are used to modify the end of the PEG chain (Suzuki et al., 2008; Yamada et al., 2008; Fondell et al., 2010; Elbayoumi et al., 2080).

It has been reported that tumor endothelial cells are different from normal endothelial cells (Hida et al., 2008). Angiogenesis has an important role in tumor growth and metastasis. Therefore, anti-angiogenic therapy by targeting tumor endothelial cells has been an attractive and important strategy in cancer therapy. In the present study, CD13 was employed as the target molecule, since it is a known tumor endothelial marker. It has been reported that oligopeptides containing a NGR motif have the ability to recognize CD13, in which the NGR motif is conjugated directly to a chemotherapeutic drug or is attached to the PEGylated liposomes (van Hensbergen et al., 2002; Pastorino et al., 2003). However, as shown in Fig. 2

and Table 3, the effect of a single-modified NGR on the cellular uptake of PEGylated liposomes was minor or negligible, even in the case of CD13 positive cells. These results provide support for the conclusion that a specific ligand can provide targeting capability to liposomes but does not necessarily result in large amounts of liposomes being taken up by the target cells. To generate further therapeutic efficacy, attempts to develop dual-targeting systems that are mediated by two different kinds of targeting-ligands were made (Saul et al., 2006; Murase et al., 2010). In fact, the dual-targeting system has the potential to show more efficient pharmacological effects, not only in vitro but also in vivo than the single modified version.

Although the properties of CPPs suggest that they would be highly promising for in vitro delivery, there are serious disadvantages for in vivo systemic applications because of non-specific interactions between the cationic charge and biological components, resulting in inactivated or collapsed liposomal formulations. Therefore, the development of a strategy for a design that can control the properties of CPPs in an in vivo situation should confer additional potential for them when used for active targeting systems. In this study, we describe the development of a dual-ligand system mediated by specific ligands and CPPs, which results in enhanced selectivity and a more efficient cellular uptake, as illustrated in Fig. 5.

In the case of a dual-targeting system modified with two different kinds of specific ligand,

when two target receptors or molecules share the same endosomes or uptake route, the specificity and affinity of the dual-targeting system for the target cells appears to be synergistically increased. As a result, the amount of cellular uptake would be expected to be increased. On the other hand, when the uptake routes are mediated by two different kinds of ligand are different, it is likely that the effect of dual targeting for cellular association and uptake would be additive or minor. The pathway for specific ligands is mainly receptor mediated endocytosis which is saturable, thus limiting the amount of liposomes that can be taken up. Furthermore, when the target molecule does not trigger the cellular uptake, no targeting system internalizes cells, which results in a negligible pharmacological effect of the cargos. As mentioned above, the fate of cellular uptake for a dual-targeting system depends on the properties of the target molecules. In the case of a dual-ligand system modified with a specific ligand and a CPP, the cellular association of the system is dependent on the nature of the specific ligand. Subsequent cellular uptake is triggered not only by the specific interaction between the ligand and target molecules, but also by non-specific uptake mediated by the CPP ligand. It is quite likely that the magnitude of the uptake mediated by CPPs is larger than that mediated by specific ligands. Therefore, a characteristic of the cellular uptake of the dual-ligand system is that it would be unsaturated and independent of the properties of the target molecules.

According to the results shown in Fig.1, STR-R4 appears to be favorable for use as a CPP

ligand because it is not functional in the presence of PEG, presumably due to masking by the aqueous layer of PEG. The calculated length of R4 and R8 was determined to be 1.63 and 3.41 nm, respectively. The predicted depth of the PEG layer is around 3 nm in a mushroom configuration (Immordino et al., 2006). Based on the calculations, it is quite likely that R4 on the surface is fully masked by the PEG layer, but that R8 is only partially masked.

The dual-ligand system modified with NGR and STR-R4 (NGR-PEG/R4-LP) exhibits a synergistic function on cellular uptake in MS-1 model cells in which CD13 is expressed (Fig. 4a). The cellular uptake of PEG-unmodified R4-LP was increased by 1.7-fold. In the presence of 10 mol% PEG, PEG/R4-LP showed a slightly lower uptake (0.9-fold), and the cellular uptake of single ligand NGR-PEG-LP decreased to around half of the LP (0.6-fold) compared with LP, respectively (Table 3). Although either NGR or STR-R4 modification in PEGylated liposomes failed to increase their cellular uptake, the dual modification of NGR and STR-R4 (NGR-PEG/R4-LP) at 10 mol% PEG-lipid stimulated uptake by 2.7-fold compared with LP. These results clearly indicate that the observed synergistic effect can be attributed to the dual-ligand formulation. Further evaluations were performed using TE cells, primary cultured tumor endothelial cells isolated from melanoma tumor xenograft mice. Even though the effect was lower than that observed for MS-1 cells, the dual-ligand system also functioned in TE cells. Therefore, this system has the possibility to be functional in in vivo tumor endothelial cells.

## 5. Conclusion

In the present study, we successfully developed a dual-ligand delivery system mediated by a specific ligand and a CPP ligand. Based on the design of the dual-ligand, STR-R4 appeared to be a suitable CPP. In the presence of PEG, modification of either specific the NGR ligand or STR-R4 had only a minor effect on cellular uptake, while the dual-modification of NGR and STR-R4 resulted in an enhanced uptake due to synergistic effects in CD13 positive cells. The proposed strategy holds considerable promise for the further development of in vivo applicable systems.

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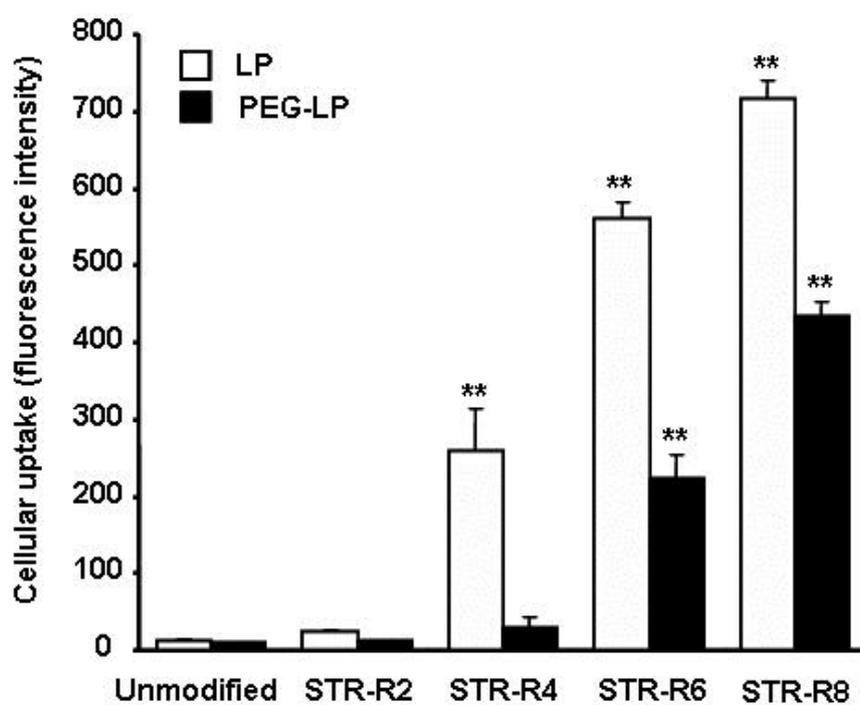
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## Figure legends

**Figure 1 Effect of STR-RX on the cellular uptake**

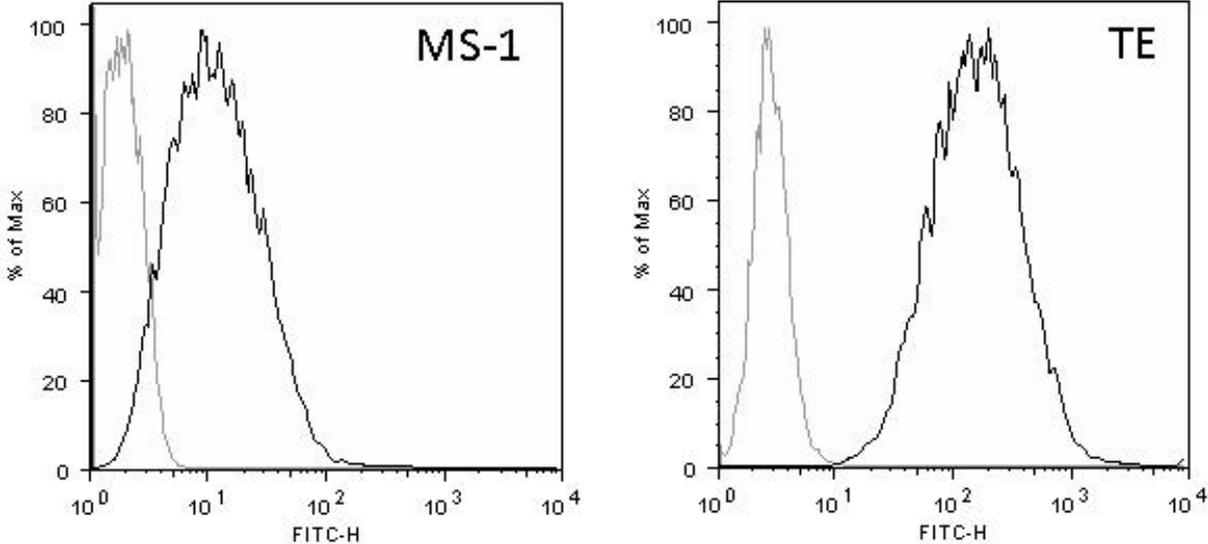
TE cells were incubated with the indicated formulations for 3 hr, and cellular association then evaluated, as described in Materials and methods. The data are expressed as the mean  $\pm$  SD (n=3). Comparisons between LP and PEG-LP were determined by the Student's *t*-test.

\*\**P*<0.01.



**Figure 2 CD13 expression on MS-1 and TE cells**

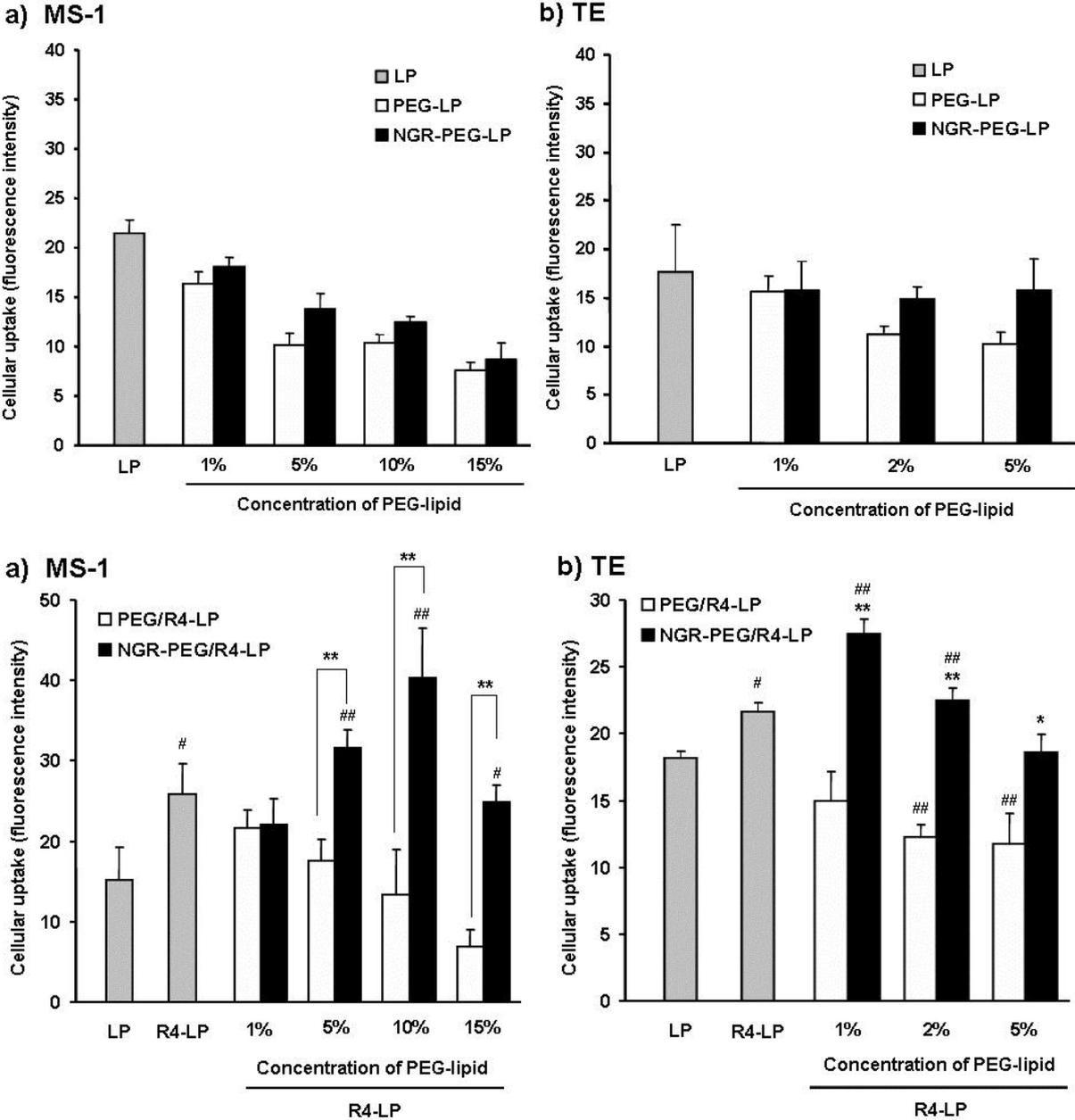
The expression of CD13 on MS-1 and TE cells was confirmed by flow cytometry analysis as described in materials and methods. Gray lines indicate non-treatment, and black lines indicate the results with antibody treatment.



**Figure 3 Cellular uptake of single-ligand liposomes.**

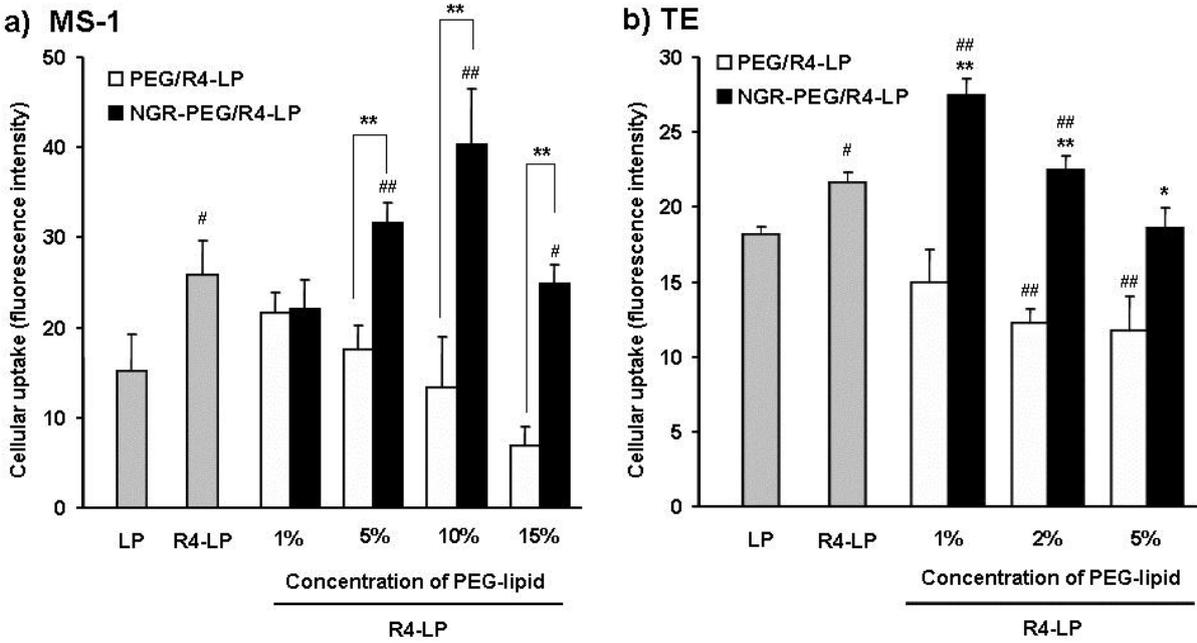
Different formulations of liposomes were incubated with (a)MS-1 cells or (b)TE cells for 3 hr and the amount of cellular uptake was determined as described in Material and methods.

Cellular uptake is expressed as mean  $\pm$  SD (n=3).



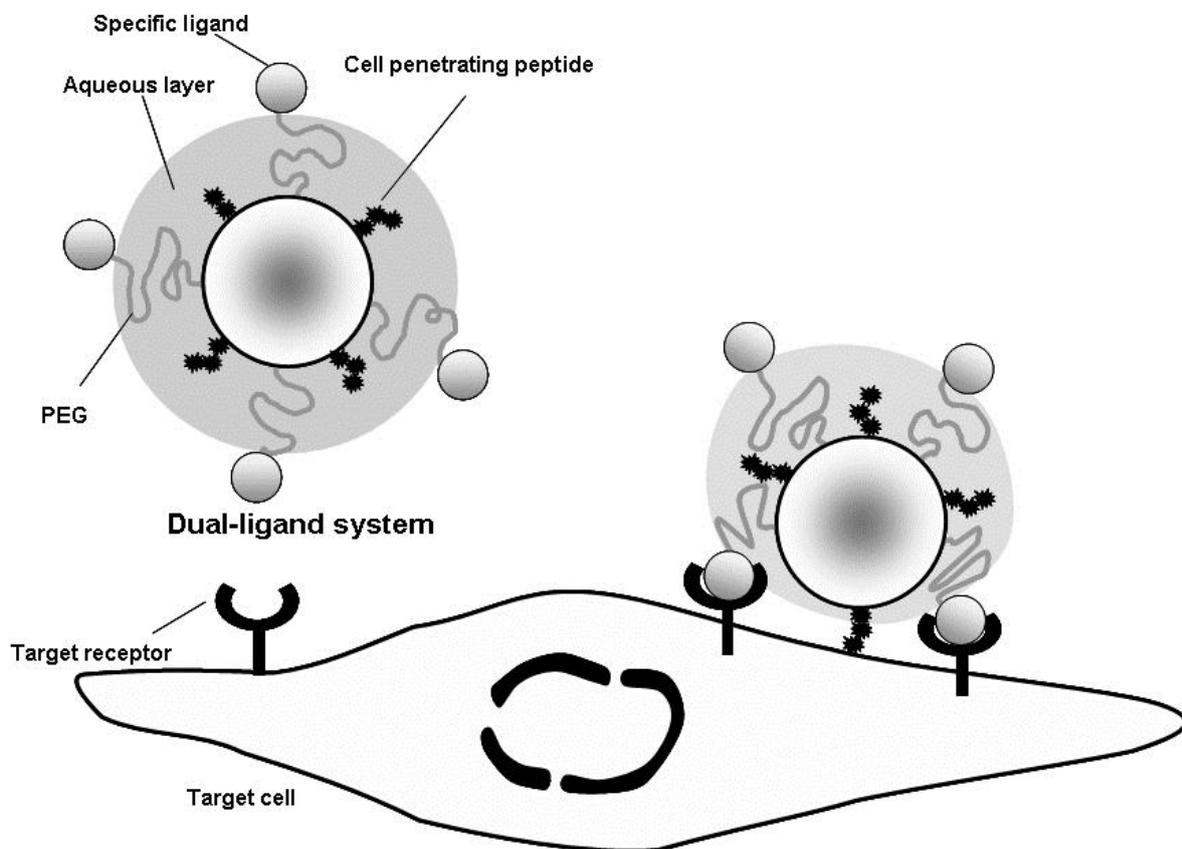
**Figure 4 Cellular uptake of dual-ligand liposomes**

Different formulations of liposomes were incubated with (a)MS-1 cells or (b)TE cells for 3 hr and the amount of cellular uptake was determined as described in Materials and methods. Cellular uptake is expressed as mean  $\pm$  SD (n=3). The statistical differences v.s. LP were determined by one-way ANOVA followed by Dunnett test. # $P$ <0.05, ## $P$ <0.01. Comparisons between PEG and NGR-PEG were determined by Student's  $t$ -test. \* $P$ <0.05, \*\* $P$ <0.01.



**Figure 5 Schematic diagram illustrating a strategy of the dual-ligand system mediated by specific ligands and CPPs.**

CPPs should not be functional and free from opsonins due to steric hindrance of the PEG layer in the systemic circulation. While after arriving at the target site, cellular association via specific ligands results in CPPs exert their powerful ability to internalize the liposomes into target cells due to the close proximity of the liposomes to the surface of target cells.



## Tables

Table 1 Physical properties of liposomes and PEGylated liposomes with STR-RX

Type of STR-RX	Modification of PEG			
	-		+	
	size (nm)	zeta-potential (mV)	size (nm)	zeta-potential (mV)
Unmodified	98±8	-3±2	102±6	-1±1
STR-R2	98±7	18±3	101±9	0±0
STR-R4	98±6	32±3	102±10	11±6
STR-R6	97±7	42±8	97±6	21±5
STR-R8	98±6	45±4	99±5	33±4

The data are expressed as mean ± SD. (n=3)

**Table 2 Physical properties of single- or dual-ligand liposomes**

Conc. of PEG-lipid	PEG-LP		NGR-PEG-LP		PEG/R4-LP		NGR-PEG/R4-LP	
	Size (nm)	zeta-potential (mV)	Size (nm)	zeta-potential (mV)	Size (nm)	zeta-potential (mV)	Size (nm)	zeta-potential (mV)
0%	97±5	0.2±0.1	-	-	89±5	2.2±1	-	-
1%	101±2	0.2±0.2	103±1	-1.6±0.9	91±8	0.8±0.5	91±8	1.3±0.8
2%	100±6	-5.0±2.4	103±8	1.5±0.7	93±2	-0.1±0.1	99±3	0.8±0.6
5%	103±8	-0.8±0.1	102±2	-0.8±0.7	92±3	-0.2±0.1	91±2	-0.1±0.1
10%	100±5	-1.5±0.4	102±6	-2.5±0.8	90±4	-1.6±0.3	88±3	-0.9±0.1
15%	100±4	-2.1±0.5	99±2	-3.6±0.6	85±7	-3.2±0.6	93±6	-1.2±0.8

The data are expressed as mean ± SD. (n=3)

The conc. of PEG-lipid 0% means PEG-unmodified formulations (LP or R4-LP).

**Table 3** Relative comparisons of cellular uptake obtained by the indicated formulations in

MS-1 cells and TE cells

Cell line : MS-1		STR-R4(-)		STR-R4(+)	
		PEG	NGR-PEG	PEG	NGR-PEG
	0	100%		170%	
Concentration of PEG (mol%)	1	76%	84%	142%	146%
	5	47%	63%	115%	210%
	10	48%	58%	88%	266%
	15	35%	40%	45%	164%

Cell line : TE		STR-R4(-)		STR-R4(+)	
		PEG	NGR-PEG	PEG	NGR-PEG
	0	100%		118%	
Concentration of PEG (mol%)	1	89%	92%	82%	151%
	2	64%	84%	67%	124%
	5	58%	90%	65%	102%

The uptake amount obtained by LP without PEG and STR-R4 is set as the control.

Zero % of PEG conc. means PEG-unmodified formulations.