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Development of cell-penetrating peptide-modified MPEG-PCL diblock copolymeric nanoparticles for systemic gene delivery

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ABSTRACT

To develop a safe and efficient systemic non-viral gene vector, methoxy poly(ethylene glycol) (MPEG)/poly(ε-caprolactone) (PCL) diblock copolymers conjugated with a Tat analog through the ester or disulfide linkage were synthesized and their suitability as a systemic non-viral gene carrier evaluated. The physicochemical properties of the MPEG-PCL diblock copolymers were determined by GPC, ^{1}H NMR and FT-IR spectroscopy. The particle sizes and *in vitro* (COS7 and S-180 cells) transfection efficiencies and cytotoxicity were evaluated. Furthermore, the luciferase activity was then determined in various tissues after intravenous injection of MPEG-PCL-SS-Tat/pCMV-Luc complex into mice bearing S-180 cells. The particle sizes of the MPEG-PCL-Tat copolymers with or without pDNA were about 40 and 60 nm, respectively. The luciferase activity in COS7 cells transfected with pCMV-Luc with MPEG-PCL-ester-Tat or MPEG-PCL-SS-Tat was higher than that with pDNA only. MPEG-PCL-SS-Tat greatly increased the transfection efficiency compared to MPEG-PCL-ester-Tat in COS7 and S-180 cells. In an *in vitro* cytotoxicity test MPEG-PCL-SS-Tat did not induce any remarkable cytotoxicity. In an *in vivo* experiment, the synthesized MPEG-PCL-SS-Tat copolymers promoted the delivery and expression of pDNA into tumor tissue in tumor-bearing mice. In conclusion, this vector might be applicable as a tumor-targeting non-viral systemic gene carrier in the clinical setting.

1. Introduction

Gene therapy refers to the treatment of human diseases by transfer of therapeutic genes to specific tissues or cells in a patient (Anderson, 1992; Luo and Saltzman, 2000; Sharma et al., 2008). It has the potential to cure both inherited and acquired genetic disorders by supplying functional copies of the defective genes to the diseased cells. An ideal non-viral gene carrier would be capable of performing the multiple functions required for the precise delivery of systemically administered DNA to the nucleus of the targeted cell; these required functions include protection of the therapeutic genes in the extracellular environment, specific cell targeting, early endosomal escape, nuclear entry, and DNA release (Wiethoff and Middaugh, 2003; Putnam, 2006).

An amphiphilic block copolymer composed of hydrophilic and hydrophobic segments has the tendency to self-assemble into micelles in a selective solvent (Allen et al., 1998; Otsuka et al., 2003; Jang et al., 2006). The polymeric micelles formed through the multimolecular assembly of the block copolymer can then be utilized for core–shell type colloidal carriers for drugs and genes (Shin et al., 1998; Kakizawa and Kataoka, 2002; Kim et al., 2003). In particular, PEG has been used to improve the solubility and steric stability of many gene delivery systems including liposomes and micelles (Jang et al., 2006). It is possible to enhance the circulation time by coating the delivery system with bioinert water-compatible polymers such as PEG, which can give rise to steric stabilization of the delivery vehicle against undesirable aggregation and non-specific electrostatic interactions with the surroundings (Templeton et al., 1997; Kakizawa and Kataoka, 2002; Park et al., 2005). The surface modification of nanoparticles with PEG can extend the circulation of polymer micelle/pDNA complexes. Furthermore, keeping the size of the carriers to less than 200 nm enables them to efficiently accumulate in tumor tissue as a result of an enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986; Hatakeyama et al., 2007). The PEG-shielded polyplex structures are typically constructed by complexation of DNA with PEG-modified polycations, largely due to the strong hydrophilicity of the PEG segments, which causes a significant weakening of the binding of the polycation to DNA. Furthermore, the PEGylation of polyplexes has an

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inhibitive effect on the cellular uptake of the polyplexes (Sharma et al., 2008). Recently, many studies have demonstrated the concept of multicomponent polymer-based formulations of gene delivery complexes. These polymers incorporate one or more additional components into the PEGylated polycation platform to enhance the performance of the PEGylated polyplexes in various aspects, including cellular uptake (Gebhart and Kabanov, 2001; Alvarez-Lorenzo et al., 2005), endosomal escape of polyplexes (Oishi et al., 2006), and timely release of the encapsulated DNA into the cytosol (Bikram et al., 2004).

The uptake of macromolecules by cells can be enhanced by cell-penetrating peptides (CPP), which are cationic or amphiphilic molecules derived from various resources including the human immunodeficiency virus Tat protein and *Drosophila antennapedia* homoprotein (Yang et al., 2009). A basic domain of Tat was previously shown to be the minimal sequence responsible for cellular and nuclear uptake, mediated by potential nuclear localization sequences in the 11-amino acid epitope YGRKKRRQRRR (Rajagopalan et al., 2007). The Tat moiety has also been shown to deliver functional fusion proteins *in vivo* (Rajagopalan et al., 2007). In addition, the partially modified Tat analog peptide synthesized in our laboratory significantly promotes the transfection of pDNA and elevates immune responses on vaginal membranes (Kanazawa et al., 2008).

In the present study, we focused on the disulfide bond to release pDNA from the complex containing these vectors. A disulfide bond is stable in the extracellular environment but this bond can be cleaved rapidly by glutathione in the cytoplasm and nucleus. As a result, a vector containing a disulfide bond can be used to prepare a stable complex of pDNA having multifunctional carriers (transcellular, endosomal escape and transnuclear). The transcellular component can be rapidly released from the carrier to provide efficient gene expression inside the cells. In the present study, to incorporate pDNA into polymeric nanoparticles by physical entrapment and electrostatic interaction and to improve intracellular uptake and nuclear import of pDNA, we synthesized Tat analog-modified methoxy poly(ethylene glycol) (MPEG)/poly(ε-caprolactone) (PCL) amphiphilic block copolymers through the disulfide linkage. The particle sizes of the formed particles before and after pDNA loading were determined. In addition, we evaluated the pDNA releasing ability of the copolymers in reducing cytoplasm, the *in vitro* cytotoxicity, the transfection efficiency in the presence or absence of serum, and the passive targeting ability of MPEG-PCL-Tat/pDNA after intravenous injection into mice bearing S-180 cells.

2. Materials and methods

2.1. Materials

Plasmid DNA (pCMV-Luc) comprising a subcloned luciferase cDNA fragment at the Hind III and BamHI sites of pcDNA3.1 was amplified in *E. coli* (DH5α) and purified using an End-free Plasmid Maxi kit (Qiagen K.K., Tokyo, Japan), followed by ethanol precipitation and dilution in Tris/EDTA buffer. DNA concentration was measured based on UV absorption at 260 nm. MPEG ($Mn = 2.0 \times 10^3$), ε-caprolactone (99+%) and Tin(II) 2-ethylhexanoate were obtained from Aldrich Chemical Co (Milwaukee, WI, USA).

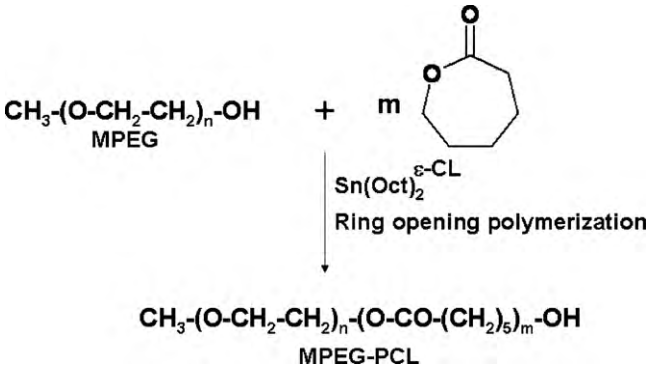
2.2. Synthesis of Tat analog

The Tat-CG analog, which consists of Cys-Gly-NH2 added to the N-terminus of HIV-Tat (48-57), and the Tat-G analog, which consists of Gly-COOH added to the N-terminus of HIV-Tat (48-57) (Table 1), were synthesized as CPP gene vectors using the Fmoc-solid-phase peptide synthesis method with the ABI 433A peptide synthesizer (Applied Biosystems, USA) as previously reported (Kanazawa et al., 2008). Both analogs were used after purification by reverse-phase HPLC. The molecular weights of the two Tat analogs were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS): Tat-CG analog, 1626.1; Tat-G analog, 1453.7.

Table 1
Structure of the Tat-G and Tat-CG analogs.

	Sequence
Tat-G	Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-COOH
Tat-CG	Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-Gly-NH2
HIV-Tat (48-57) original	Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg

Underlined sequences were modified from the natural sequence of each peptide.



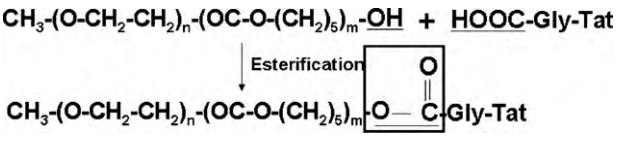
Scheme 1. Synthesis procedure of MPEG-PCL diblock polymer.

2.3. Synthesis of MPEG-PCL

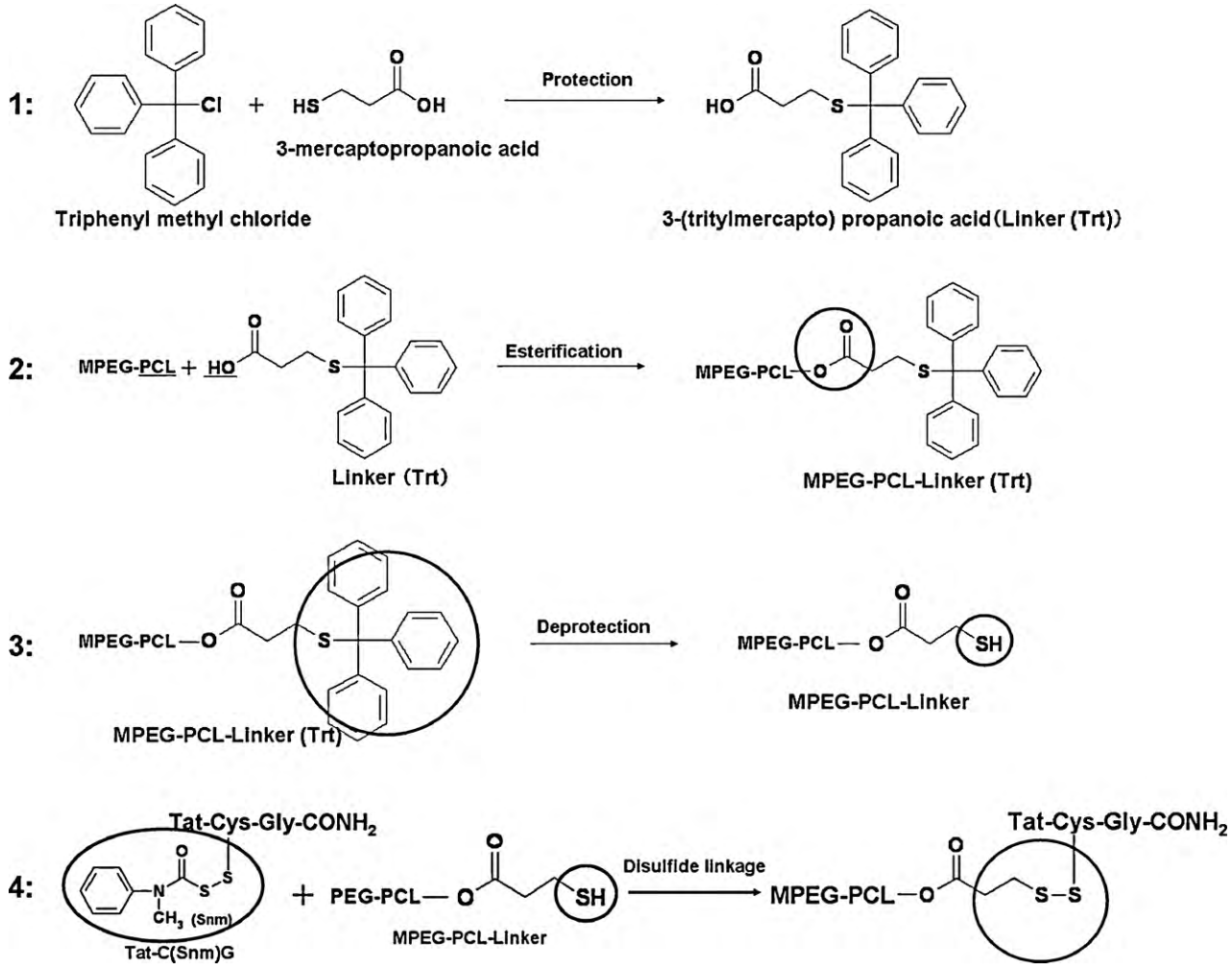
MPEG-PCL block copolymers were synthesized by ring-opening copolymerization, as illustrated in Scheme 1. Briefly, a predetermined amount of ε-caprolactone was added to a recovery flask containing MPEG and a small amount of Tin(II) 2-ethylhexanoate (0.1%, wt/wt). The recovery flask was then filled with N_2 gas, sealed off, and placed in an oil bath at 130–140 °C for 3 days. At the end of the polymerization, the crude copolymers were suspended in distilled water and centrifuged at 11,000 rpm for 3 min to remove any un-reacted monomer and oligomer. The supernatant was then filtered and dialyzed using a spectra/pore CE tube before being thoroughly freeze-dried.

2.4. Synthesis of Tat-conjugated MPEG-PCL through the ester bond

The hydroxyl group of the MPEG-PCL block copolymers was converted into a Tat analog through the ester bond. The Tat analog-conjugated MPEG-PCL via the ester bond was synthesized according to Scheme 2. Tat-G (0.02 mmol) and MPEG-PCL (0.02 mmol) were dissolved in dimethylformamide (DMF), and then water-soluble carbodiimide (WSCI, 0.02 mmol) and 4-



Scheme 2. Synthesis procedure of MPEG-PCL-ester-Tat.



Scheme 3. Synthesis procedure of MPEG-PCL-SS-Tat.

dimethylaminopyridine (0.02 mmol) were added and reacted. After 24 h, the reaction solution was evaporated and dialyzed in distilled water using a dialysis tube (3,500 MW, Spectrum Laboratories, Inc., USA) for 24 h to remove the non-reacted Tat-G, and after freeze-drying, MPEG-PCL-conjugated-Tat through the ester bond (MPEG-PCL-ester-Tat) was obtained.

2.5. Synthesis of Tat analog-conjugated MPEG-PCL through the disulfide linkage (Scheme 3)

To protect the thiol residue of 3-mercaptopropanoic acid using a Trt residue, 3-mercaptopropanoic acid dissolved in dichloromethane (DCM) was added to triphenyl methyl chloride (62.3 mmol) dissolved in DCM and stirred for 16 h, resulting in the linker (Trt). Next, MPEG-PCL (0.02 mmol) was dissolved in 1 mL of DMF and then linker (Trt) (0.02 mmol), WSCI (0.02 mmol) and 4-dimethylaminopylidine (0.02 mmol) were added. After this mixture was reacted for 24 h and dialyzed using a dialysis tube (500 MW, Spectrum Laboratories) for 24 h, MPEG-PCL-linker was obtained. Finally, MPEG-PCL-Linker (0.006 mmol) and Tat-CG (0.006 mmol) were dissolved in DMF and the disulfide linkage reaction allowed to proceed. After 24 h, the reaction solution was evaporated and dialyzed in distilled water using a dialysis tube (3,500 MW, Spectrum Laboratories) for 24 h to remove the non-reacted Tat-CG, and after freeze-drying, MPEG-PCL-conjugated-Tat through the disulfide bond (MPEG-PCL-SS-Tat) was obtained.

2.6. Characterization of synthesized MPEG-PCL and MPEG-PCL-Tat

The chemical structure and molecular weight of synthesized MPEG-PCL was determined by FT-IR, ^{1}H NMR and GPC spectroscopy. Conjugation of the Tat analog to MPEG-PCL through the ester bond or disulfide bond was confirmed using the ninhydrin reaction, which becomes reddish violet upon reacting with the amino acids. Weights were characterized by elution times relative to polyethylenglycol monodisperse standards using GPC.

2.7. Preparation of pCMV-Luc complexes

Lipofectamine® (16 μg) and pCMV-Luc (1 μg) complexes were prepared by incubating the two for 30 min at 4 °C. Complexes of pCMV-Luc (1 μg) and MPEG-PCL-ester-Tat or MPEG-PCL-SS-Tat were prepared by mixing the two components at a molecular weight ratio ranging from 1/1 to 50/1 for 30 min at 4 °C. The mixtures were then used in characterization and transfection studies.

2.8. Characterization of pCMV-Luc complexes

The mean diameter and size distribution of the pCMV-Luc complexes were measured using a DLS-700 unit (Otsuka Electronics Co., Ltd., Osaka, Japan). Zeta potential was measured using a NICOMP 380ZLS unit (Particle Sizing Systems, Shanghai, China). Character-

ization of both carrier nanoparticles complexed with or without pCMV-Luc was performed. pCMV-Luc complexed with MPEG-PCL or MPEG-PCL-Tat nanoparticle samples were analyzed by agarose gel electrophoresis in tris–borate–EDTA buffer (TBE: 40 nM tris–borate, 1 mM EDTA, pH 7.4). Various ratios of copolymers to pDNA samples were loaded onto a 1% agarose gel containing ethidium bromide (0.5 mg/mL) and electrophoresed in TBE buffer (0.25%) at 100 V for 40 min. The gel was visualized on a UV illuminator to determine the location of the pDNA.

2.9. In vitro transfection

COS7 (African green monkey kidney epithelial-like cells) and sarcoma-180 (S-180) cells were maintained at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Invitrogen Co., USA) and 1% penicillin/streptomycin (stock 10,000 U/mL, 10,000 mg/mL, Invitrogen Co.). COS7 and S-180 cells (5×10^5 cells) were seeded in 6-well culture plates, and after a 24-h incubation in DMEM containing 10% FBS (70–80% confluence), the cells were washed with PBS and then 1.9 mL of culture medium (Opti-MEM) without FBS was added to each well. pCMV-Luc complex solution (100 μL containing pCMV-Luc: 1 μg) was applied to each well. After 4 h, the medium was removed and replaced with 10% FBS containing DMEM for a further 24-h incubation. The cells were then washed three times with PBS, lysed by addition of 100 μL of lysis buffer per well, and left to stand for 15 min at room temperature. Cell lysates were then collected and centrifuged at 15,000 rpm for 3 min. After the addition of 20 μL of luciferase substrate solution to 40 μL of cell lysate, the luciferase activity was measured using a chemiluminescence detector (MicroLumat Plus LB96V; Berthold, Germany). The total protein in the cells was determined using a standard Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). A 5-μL aliquot of cell lysate diluted 160 times with ultrapure water was then reacted with 40 μL of dye reagent for 1 h at room temperature, and the absorbance was measured at 595 nm using a microplate reader (Tecan Safire, Tecan Trading AG, Switzerland). The protein concentration of cell lysate was calculated using a calibration curve for BSA standards (2 mg/mL). The results are shown as relative light units (RLU) per mg of protein.

2.10. In vitro cytotoxicity

COS7 cells (25,000 cells/well) with 100 μL of DMEM containing 10% FBS were seeded into 96-well plates and incubated for 24 h. The cells were then washed three times with PBS and transfected with the MPEG-PCL-SS-Tat/pCMV-Luc complex at a ratio of 1/1 to 50/1 for 4 h. The cells were then washed three times with PBS and cultured in DMEM containing 10% FBS for 20 h, after which 10 μL of CCK-8 solution was added to each well. After 4 h, the absorbance was measured at 450 nm using a microplate reader. The absorbance of control cells indicated 100% cell viability.

2.11. In vitro transfection at 4 °C to inhibit endocytosis

COS7 cells (5×10^5 cells) were seeded in 6-well culture plates and incubated for 23 h in DMEM containing 10% FBS. The cells were washed twice with PBS and then 1.9 mL of culture medium (Opti-MEM) without FBS was added to each well for a 1-h incubation at 4 °C. pCMV-Luc complex solution (100 μL containing 1 μg pCMV-Luc) was then applied to each well for a 4-h incubation at 4 °C. The medium was then removed and replaced with 10% FBS containing DMEM for further incubation for 20 h at 37 °C, after which luciferase activity was determined.

2.12. Effects of various inhibitors on pCMV-Luc complex uptake

COS7 cells (5×10^5 cells) were seeded in 6-well culture plates and incubated for 24 h in DMEM containing 10% FBS. The cells were then washed with PBS twice and 1.9 mL of culture medium (Opti-MEM) without FBS but containing the following inhibitors was added to each well for 30 min: amiloride (3 mM) as a macropinocytosis inhibitor, filipin (1 mg/mL) as a caveola-mediated pathway inhibitor, and chlorpromazine (10 μg/mL) as a clathrin-mediated pathway inhibitor. pCMV-Luc complex solution (100 μL containing 1 μg of pCMV-Luc) was applied to each well and after 4 h the medium was removed and replaced with 10% FBS containing DMEM for further incubation for 20 h at 37 °C, after which luciferase activity was determined.

2.13. In vivo study

S-180 cells were cultured to 70–80% confluence in DMEM containing 10% FBS and 1% penicillin/streptomycin (stock 10,000 U/mL, 10,000 mg/mL, Invitrogen Co.). Eight-week-old ICR female mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Tumors were generated by subcutaneously injecting 5×10^6 S-180 cells suspended in 300 μL of FBS(−)RPMI medium into the backs of the mice using a syringe attached to a 23-G needle. Tumor size was measured using a Vernier caliper across the longest and shortest diameter (mm) and tumor volume was calculated using the following equation: tumor volume (mm^3) = longest diameter × shortest diameter^2 × 1/2. After 20 days, when tumor size reached above 100 mm^3, the mice received an intravenous injection of MPEG-PCL-SS-Tat/pCMV-Luc complexes in 100 μL ultrapure water. After 24 h, the mice were sacrificed and the lung, heart, liver, spleen, kidney and tumor tissues collected. Thereafter, the tissues were homogenized in lysis buffer at a volume-to-weight ratio of 4 mL lysis buffer per gram of each organ, and processed three times by freezing (−80 °C) and thawing. Each homogenate was then centrifuged at 14,000 rpm for 15 min, and the luciferase activity in 20 μL of supernatant was measured in a similar manner to the *in vitro* transfection.

2.14. Statistical analysis

The data from the *in vitro* experiments expresses the mean ± S.D. The data from the *in vivo* experiments expresses the mean ± S.E. Statistical analysis of the data was performed using an unpaired Student's *t*-test for two groups. Statistical significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{n.s.}P > 0.05$.

3. Results

3.1. Synthesis and characterization of MPEG-PCL and MPEG-PCL-Tat

MPEG-PCL block copolymers were synthesized by ring-opening polymerization of ε-CL in the presence of MPEG with a small amount of catalyst. The FT-IR and proton NMR spectra for a typical MPEG-PCL copolymer are shown in Fig. 1. The MPEG-PCL copolymer clearly exhibited peaks characteristic of both PEG and PCL. The absorption band at 1725 cm^{-1} was attributed to C=O stretching vibrations of ε-CL. The absorption bands at 2875 and 2937 cm^{-1} were attributed to C–H stretching vibrations of MPEG and ε-CL, respectively. The ^{1}H NMR spectrum of the MPEG-PCL block copolymer is shown in Fig. 1b. The peaks at 1.27–1.31, 1.51–1.56, 2.25–2.28 and 3.96–3.99 ppm were assigned to the methylene protons in the PCL units. The peak at 3.5 ppm was attributed to the methylene protons of the PEG units. The ratio of the 1.27–1.31, 1.51–1.56, 2.25–2.28, 3.5 and 3.96–3.99 peak areas was determined

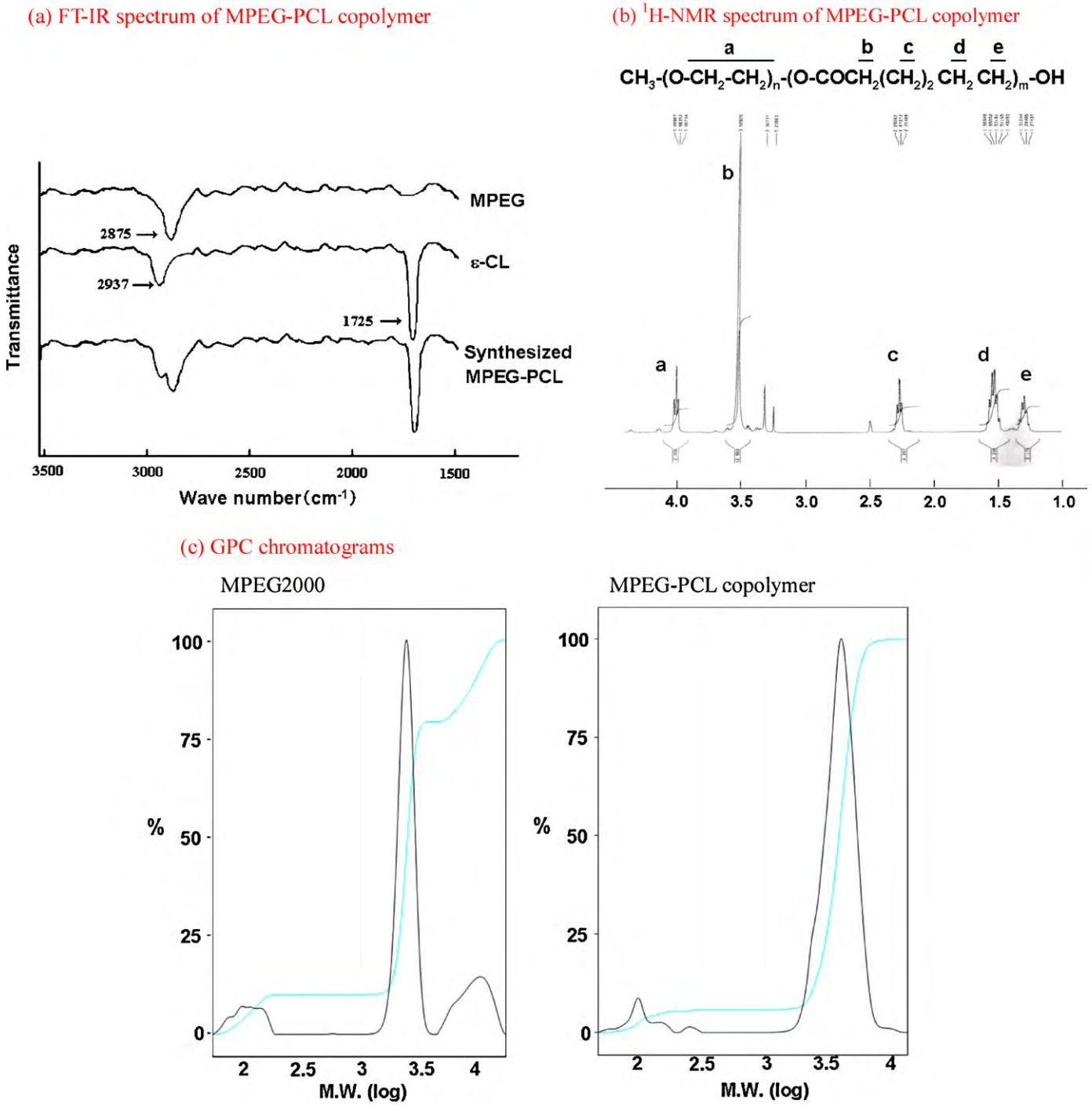


Fig. 1. Characterization of synthesized MPEG-PCL by FT-IR and ^{1}H NMR: (a) FT-IR spectrum, (b) ^{1}H NMR spectrum and (c) GPC chromatograms of MPEG2000 and MPEG-PCL copolymer.

to be 1:2:1:6:1. The GPC chromatograms of MPEG-PCL copolymer were displayed in Fig. 1c. The composition and molecular weights of MPEG-PCL, measured using ^{1}H NMR and GPC, are shown in Table 2. The molecular weights determined by GPC are mostly in reasonable agreement with those calculated based on the peak integrations in the ^{1}H NMR data. In addition, conjugation of the Tat analog to MPEG-PCL through the ester or disulfide bond was confirmed using the ninhydrin reaction, which becomes reddish violet upon reacting with the amino residue.

The particle size and zeta potential of MPEG-PCL, MPEG-PCL-ester-Tat and MPEG-PCL-SS-Tat in distilled water is shown in Table 3. The particle size of the MPEG-PCL micelles was larger

Table 2
Molecular weight of the synthesized MPEG-PCL block copolymer.

	MPEG *Mn*	mol% of ε-CL		Mn[a]	Mn[b]	Mw/Mn[b]
		Feed	Product[c]			
MPEG-PCL	2,000	20	17	3,940	3,718	1.08

[a] Number-average molecular weight calculated from the ^{1}H NMR spectrum.
[b] Number-average molecular weight (Mn) and weight-average molecular weight (Mw) measured by GPC (calibrated with PEG standard).
[c] Estimated from the integral height of hydrogen shown in the ^{1}H NMR spectrum.

Table 3
Mean particle diameter and zeta potential of synthesized MPEG-PCL, MPEG-PCL-ester-Tat and MPEG-PCL-SS-Tat.

	Mean particle diameter (nm)	Zeta potential (mV)
MPEG-PCL	73.8 ± 5.0	−2.06 ± 0.8
MPEG-PCL-ester-Tat	30.1 ± 12.8	+6.78 ± 1.6
MPEG-PCL-SS-Tat	31.1 ± 15.9	+6.12 ± 0.5

Mean ± S.D., $n = 3$.

than that of the Tat analog-conjugated MPEG-PCL, and the zeta potential of MPEG-PCL showed a negative charge whereas that of Tat analog-conjugated MPEG-PCL showed a positive charge. The pCMV-Luc complex formation of each block copolymer as visualized by agarose gel electrophoresis is shown in Fig. 2. MPEG-PCL did not show the delay of bands of pCMV-Luc at any weight ratio. In contrast, MPEG-PCL-ester-Tat and MPEG-PCL-SS-Tat showed a delay of bands of pCMV-Luc at up to a 5/1 weight ratio. The mean diameter and zeta potential of the MPEG-PCL-Tat/pCMV-Luc complex at various weight ratios were also determined (Table 4). The mean diameter of pCMV-Luc complexed with MPEG-PCL-ester-Tat or MPEG-PCL-SS-Tat was close to about 60 nm at up to a 5/1 weight

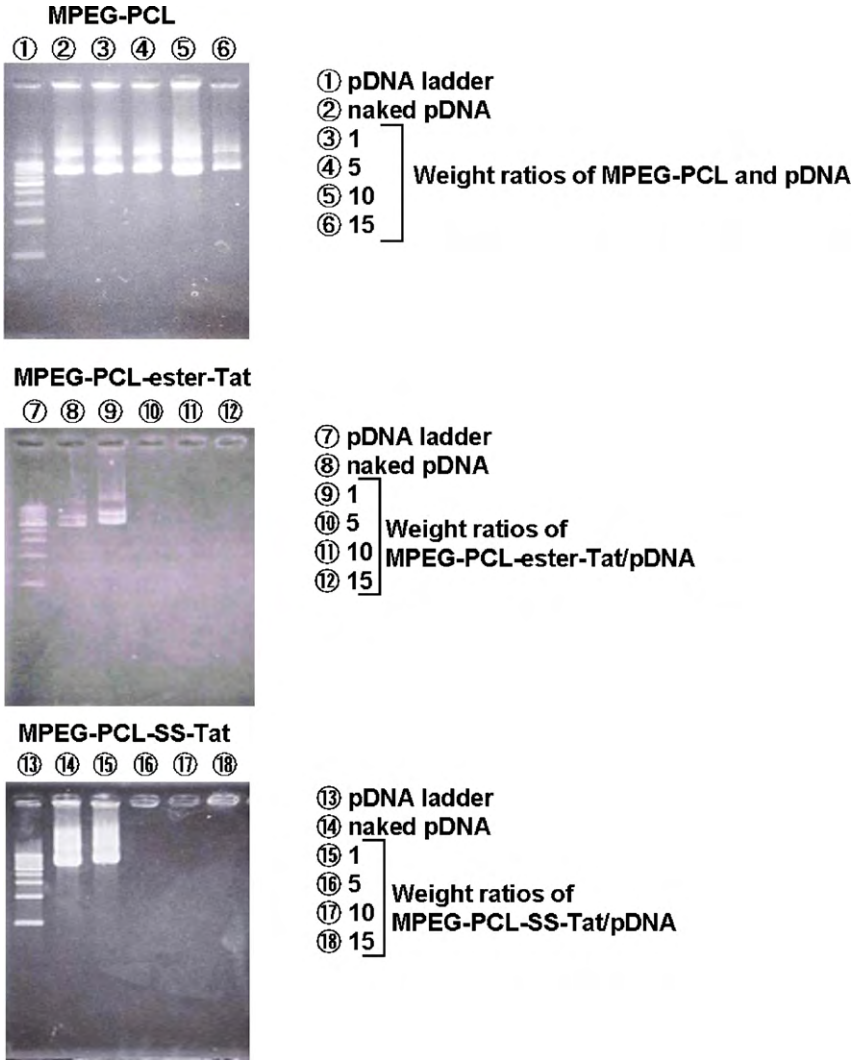


Fig. 2. Complex formation of MPEG-PCL, MPEG-PCL-ester-Tat and MPEG-PCL-SS-Tat with pCMV-Luc. The mixture of pCMV-Luc and MPEG-PCL, MPEG-PCL-ester-Tat or MPEG-PCL-SS-Tat was prepared at 4 °C for 30 min and analyzed by 1% agarose gel electrophoresis. Electrophoresis was carried out in TBE buffer at 100 mV for 40 min and the gel was then stained with ethidium bromide.

Table 4
Mean diameter and zeta potential of the MPEG-PCL-ester-Tat or MPEG-PCL-SS-Tat/pCMV-Luc complexes.

	Weight ratios of vector/pCMV-Lucr	Mean particle diameter (nm)	Zeta potential (mV)
MPEG-PCL-ester-Tat	1/1	121.6 ± 24.0	−14.4 ± 5.3
	5/1	64.9 ± 9.0	+3.6 ± 0.7
	10/1	67.2 ± 56.8	+6.3 ± 0.8
MPEG-PCL-SS-Tat	1/1	151.2 ± 8.7	−14.5 ± 1.9
	5/1	59.7 ± 9.3	+10.1 ± 0.4
	10/1	64.3 ± 8.1	+11.9 ± 1.2

Mean ± S.D., $n = 3$.

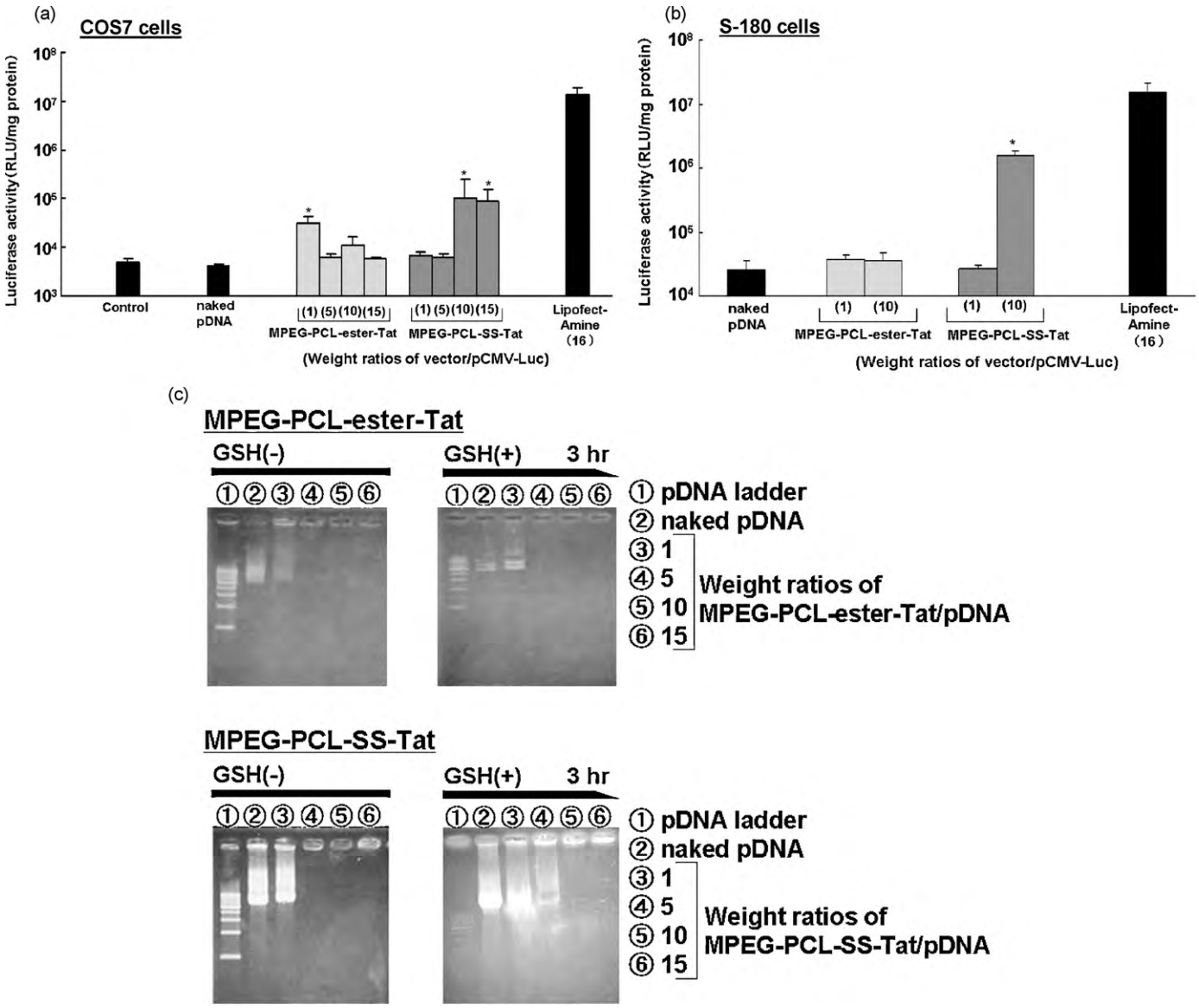


Fig. 3. Luciferase activities in COS7 and S-180 cells after transfection of Tat-conjugated MPEG-PCL polymers/pCMV-Luc and agarose gel electrophoresis of MPEG-PCL-Tat/pCMV-Luc complex treated with reducing agent. MPEG-PCL-ester-Tat (1–15 μg), MPEG-PCL-SS-Tat (1–15 μg) and lipofectamine (16 μg) complexed with 1 μg pCMV-Luc were transfected into (a) COS7 and (b) S-180 cells for 4 h. After a 20-h incubation, luciferase activity in COS7 cells was determined by the luciferase assay. Each bar represents the mean ± S.D. ($n = 3$). $^*P < 0.05$ versus naked pDNA. (c) Each sample was treated with a solution of GSH (10 mM) at 37 °C for 3 h and analyzed by 1% agarose gel electrophoresis. Electrophoresis was carried out in TBE buffer at 100 mV for 40 min and the gel then stained with ethidium bromide.

ratio. The zeta potential of these complexes at a 1/1 weight ratio showed a negative charge whereas that at 5/1 and 10/1 weight ratios showed a positive charge.

3.2. Transfection efficiency of MPEG-PCL-Tat in COS7 cells

The luciferase activity of the MPEG-PCL-Tat and pCMV-Luc complexes in COS7 at the various weight ratios is shown in Fig. 3. MPEG-PCL-ester-Tat significantly induced luciferase activity at a 1/1 weight ratio compared to naked pCMV-Luc. In contrast, MPEG-PCL-SS-Tat significantly increased transfection efficiency at 10/1 and 15/1 weight ratios compared to naked pCMV-Luc in COS7 cells. However, the luciferase activity in COS7 cells transfected with MPEG-PCL-ester-Tat/pCMV-Luc complexes at high weight ratios (5/1 to 10/1) decreased.

The luciferase activity of S-180 cells transfected with pCMV-Luc MPEG-PCL-ester-Tat or MPEG-PCL-SS-Tat complexes and Lipofectamine® was also determined (Fig. 3b). The luciferase activity of S-180 cells did not increase with MPEG-PCL-ester-Tat and was similar to that of naked pCMV-Luc. On the other hand, MPEG-PCL-SS-Tat greatly elevated the transfection efficiency compared to MPEG-PCL-ester-Tat. The gel electrophoresis analysis of MPEG-PCL-Tat and pCMV-Luc complexes after treatment for 3 h with the reducing reagent, 10 mM glutathione, which is similar to the physiological concentration in the cytosol, is shown in Fig. 3c. At the weight ratios of 5/1 and 10/1 of PEG-PCL-SS-Tat and pDNA, bands of released pCMV-Luc were observed. However, released pCMV-Luc bands were not observed with MPEG-PCL-ester-Tat.

3.3. Evaluation of MPEG-PCL-SS-Tat as an efficient gene carrier

The luciferase activity of COS7 cells transfected with Tat analog, MPEG-PCL-SS-Tat and Lipofectamine® in the presence of serum is shown in Fig. 4a. The luciferase activity following transfection with pCMV-Luc alone or Tat analog/pDNA complex did not increase, similar to the non-treated control cells. However, MPEG-PCL-SS-

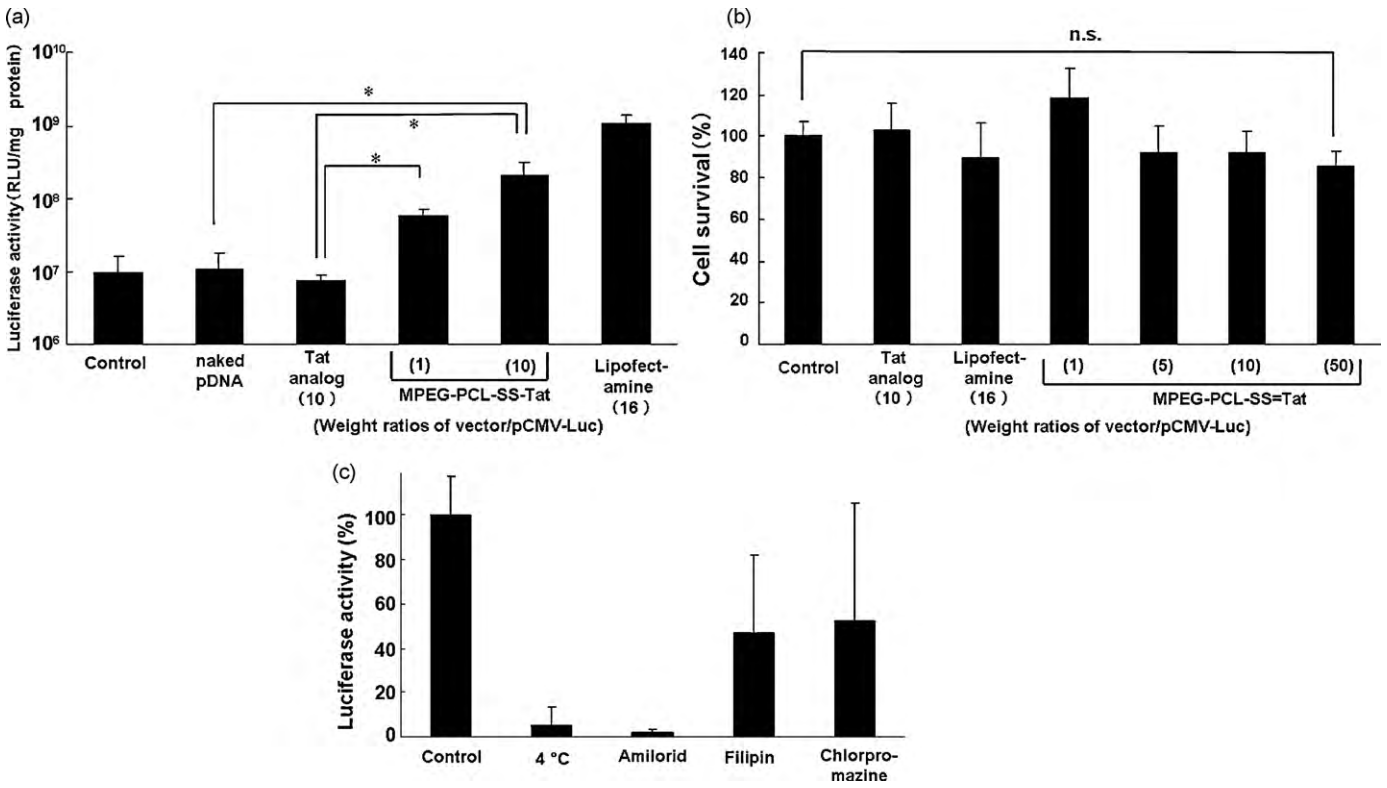


Fig. 4. Luciferase activities in COS7 cells with serum or several inhibitors and *in vitro* cytotoxicity after transfection of MPEG-PCL-SS-Tat/pCMV-Luc complexes. (a) MPEG-PCL-SS-Tat (1 and 10 μg), Tat analog (10 μg) and lipofectamine (16 μg) complexed with pCMV-Luc (1 μg) were transfected into COS7 cells for 4 h in the presence of serum. After a 20-h incubation, luciferase activity in COS7 cells was determined by the luciferase assay and (b) MPEG-PCL-SS-Tat (1–50 μg), Tat analog (10 μg) and lipofectamine (16 μg) complexed with pCMV-Luc (1 μg) were transfected into COS7 cells for 4 h. After a 20-h incubation, cytotoxicity in COS7 cells was measured by the WST-8 assay. (c) MPEG-PCL-SS-Tat (10 μg) complexed with pCMV-Luc (1 μg) was transfected into COS7 cells for 4 h. Before transfection, COS7 cells were treated by incubation for 30 min at 4 °C, amiloride (3 mM), filipin (1 μg/mL) or chlorpromazine (10 μg/mL). After a 20-h incubation, luciferase activity in COS7 cells was determined by the luciferase assay. Each bar represents the mean ± S.D. ($n = 3$). $^{*}P < 0.05$; $^{n.s.}P > 0.05$.

Tat significantly increased luciferase activity dose-dependently, after complexation with pCMV-Luc, even in the presence of serum, compared to naked pDNA and Tat analog complex. Furthermore, evaluation of the *in vitro* cytotoxicity of pCMV-Luc complexed with MPEG-PCL-SS-Tat at various weight ratios, using the WST-8 assay (Fig. 4b), revealed no cytotoxicity of MPEG-PCL-SS-Tat at any concentration.

In order to determine the mode of cellular uptake of pDNA complexed with MPEG-PCL-SS-Tat, COS7 cells were incubated with the following inhibitors for 30 min before transfection with the various complexes and luciferase activity then determined: 4 °C transfection for endocytosis inhibition, amiloride as a macropinocytosis inhibitor, filipin as a caveola-mediated pathway inhibitor and chlorpromazine as a clathrin-mediated pathway inhibitor. As shown in Fig. 4c, the luciferase activity markedly decreased in the presence of each inhibitor. In particular, amiloride and transfection at 4 °C strongly and significantly inhibited gene expression.

3.4. *In vivo* transfection of MPEG-PCL-SS-Tat

The luciferase activity in various tissues, including the tumor, 24 h after intravenous injection into the tumor-bearing mice is shown in Fig. 5. In the naked pCMV-Luc-injected mice, the luciferase activity had not advanced above the control level in any tissue. In contrast, when the MPEG-PCL-SS-Tat/pCMV-Luc (10/1) complex was injected into the mice, the luciferase activity in the lung, liver, spleen, kidney and tumor was significantly higher than produced in each tissue by the naked pCMV-Luc-injected mice. In particular, the luciferase activity in tumor tissue after intravenous injection of MPEG-PCL-SS-Tat/pCMV-Luc complex was significantly higher than that in the other normal tissues.

4. Discussion

An ideal non-viral gene carrier can perform the multiple functions required for the specific and precise delivery of systemically administered DNA to the nucleus of the targeted cell. In this study, in order to develop a systemic non-viral gene delivery carrier, methoxy poly(ethylene glycol) (MPEG)/polycaprolactone (PCL) diblock copolymers conjugated with a Tat analog through the ester or disulfide linkage were prepared and their transfection efficiency into tumor cells and tissue then evaluated. MPEG-PCL block copolymers were synthesized by ring-opening polymerization of ε-CL in the presence of MPEG with a small amount of catalyst.

FT-IR and proton NMR spectra for a typical MPEG-PCL copolymer clearly indicate that the MPEG-PCL copolymer exhibits peaks characteristic of both PEG and PCL (Fig. 1). The molecular weight of MPEG-PCL was measured using ^{1}H NMR and GPC. The GPC chromatograph of MPEG-PCL showed unimodal distribution. The composition and molecular weights of MPEG-PCL, measured using peak integrations in the ^{1}H NMR and GPC, are shown in Table 2. The molecular weights detected by GPC are mostly in reasonable agreement with those calculated based on the ^{1}H NMR data. In addition, conjugation of the Tat analog to MPEG-PCL through the ester or disulfide bond was confirmed using the ninhydrin reaction. The particle size of the MPEG-PCL micelles was larger than that of the Tat analog-conjugated MPEG-PCL, and the zeta poten-

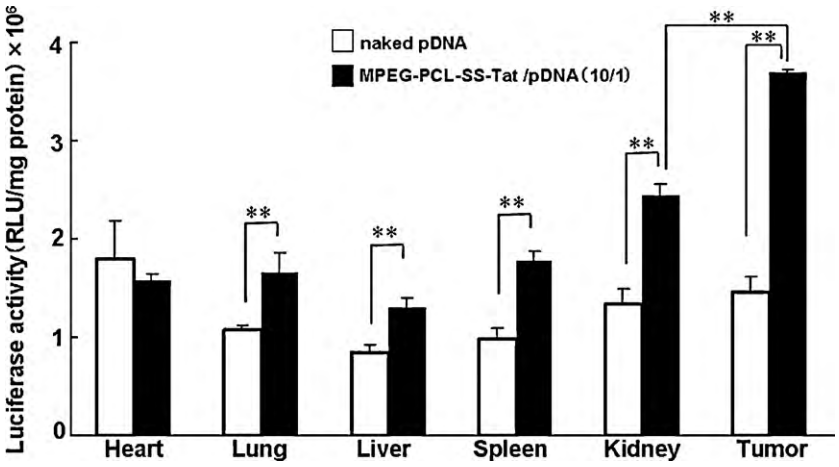


Fig. 5. Luciferase activity in various tissues 24 h after intravenous injection of MPEG-PCL-SS-Tat/pCMV-Luc complex into mice bearing S-180 cells. The pCMV-Luc (50 μg)/MPEG-PCL-SS-Tat (500 μg) complex was intravenously injected into mice bearing S-180 cells. At 24 h post-injection, each tissue was harvested, homogenized and centrifuged, and the luciferase activity of the supernatant then determined by the luciferase assay. Each bar represents the mean ± S.E. ($n = 4$). $^{**}P < 0.01$.

tial of MPEG-PCL showed a negative charge whereas that of Tat analog-conjugated MPEG-PCL showed a positive charge. This suggests that these synthesized Tat-conjugated polymers can form nanoparticles, possibly like a polymer micelle, and which are different from MPEG-PCL nanoparticles and have a slightly positive charge in water, most likely because the Tat analog is presented on the surface of the nanoparticles. As shown in Fig. 2, MPEG-PCL without Tat did not show a delay of pCMV-Luc bands, indicating that MPEG-PCL cannot form a complex with pCMV-Luc at any weight ratio due to the negative charge of the MPEG-PCL surface. In contrast, MPEG-PCL-ester-Tat and MPEG-PCL-SS-Tat showed a delay of pCMV-Luc bands at up to a 5/1 weight ratio, indicating that Tat analog-conjugated MPEG-PCL can form a stable complex with pCMV-Luc.

The results of the *in vitro* transfection experiments in COS7 and S-180 cancer cells indicate that MPEG-PCL-SS-Tat also significantly increases the transfection efficiency at 10/1 and 15/1 weight ratios compared to naked pCMV-Luc. MPEG-PCL-ester-Tat did not increase luciferase activity, compared to naked pDNA (Fig. 3a and b). We believe that the decompaction in these complexes with the ester form bond was insufficient to release the pDNA in the cytosol, as shown in Fig. 3c. On the other hand, MPEG-PCL-SS-Tat markedly increased luciferase activity in both cell types compared to naked pDNA, while MPEG-PCL-ester-Tat/pCMV-Luc did so at vector/pDNA complex weight ratios of 10/1 or higher. In order to achieve efficient transcription, it is very important that pDNAs are able to be released from the vector once inside the cell. The disulfide bond is important for gene delivery because of the decondensation and release of pDNA that is expected to be triggered by the reducing environment of the cytosol and nucleus. As shown in Fig. 3c, bands of released pCMV-Luc were observed at the weight ratios of PEG-PCL-SS-Tat to pDNA of 5/1 and 10/1 in the gel electrophoresis analysis of MPEG-PCL-Tat and pCMV-Luc complexes after treatment for 3 h with the reducing regent, 10 mM glutathione. These results suggested that MPEG-PCL-ester-Tat and MPEG-PCL-SS-Tat formed nano-sized complex with pDNA due to not only the electrostatic interaction by Tat but also constructive and physical complexation by micelles. So, in the reductive environment, the interaction between MPEG-PCL-SS-Tat and pDNA was reduced because the ability of constructive and physical complexation was disappered by cleavage disulfide bonds. As a result, pDNA was easily to release from MPEG-PCL-SS-Tat.

In order for a systemic gene carrier to be effective, the degradation that occurs by interaction with serum proteins and enzymes needs to be avoided. Thus, the luciferase activity in COS7 cells transfected with Tat analog, MPEG-PCL-SS-Tat and Lipofectamine® in the presence of serum was determined (Fig. 4a). MPEG-PCL-SS-Tat after complexation with pCMV-Luc significantly and strongly increased luciferase activity dose-dependently, even in the presence of serum, compared to naked pDNA and Tat analog complex. This is most likely due to nonionic water-soluble PEG chains acting as a hydrophilic shell to stabilize and protect the pDNA from serum proteins. Consequently, a number of MPEG-PCL-SS-Tat molecules might survive to then produce systemic transfection in the presence of serum. Based on the *in vitro* cytotoxicity tests of pCMV-Luc complexed with MPEG-PCL-SS-Tat at various weight ratios using the WST-8 assay (Fig. 4b), MPEG-PCL-SS-Tat does not induce any remarkable cytotoxicity at any ratios of vector/pDNA. These findings indicate that the MPEG-PCL-SS-Tat synthesized in this study would be suitable as a systemic non-viral gene carrier.

Most non-viral vectors are internalized by endocytosis such as macropinocytosis, the clathrin-mediated pathway, the caveola-mediated pathway and fusion. Gene expression efficiency is strongly related to the cellular uptake pathway of genes. Therefore, determination of the cellular uptake pathway of MPEG-PCL-SS-Tat was required. As shown in Fig. 4c, the luciferase activity markedly decreased in the presence of each inhibitor. In particular, amiloride and transfection at 4 °C strongly and significantly inhibited gene expression, suggesting that MPEG-PCL-SS-Tat mainly transfects the gene through the macropinocytosis pathway. As already reported (Khalil et al., 2006; Futaki et al., 2007; Nakase et al., 2007; Chang et al., 2007, 21–24), arginine-rich peptides such as Tat and oligoarginine mainly pass into the cytosol through this macropinocytosis pathway. Thus, MPEG-PCL-SS-Tat/pDNA nanoparticles might also undergo uptake into the cell predominantly through macropinocytosis via the Tat analog, which may be present on the surface of these nanoparticle complexes. Furthermore, these results suggest that a marked proportion of these nanoparticles were transfected through the other endocytosis processes, via the caveola- and clathrin-mediated pathways.

The transfection of pCMV-Luc into tumor tissue inoculated with S-180 cells increased 24 h after intravenous injection of the naked pCMV-Luc or MPEG-PCL-SS-Tat/pCMV-Luc complex (Fig. 5). In the naked pCMV-Luc-injected mice, the luciferase activity did not advance above the control level in any tissue, possibly due to degradation by nucleases in the serum. In contrast, when the MPEG-PCL-SS-Tat/pCMV-Luc complex was injected into the mice, the luciferase activity in the lung, liver, spleen, kidney and tumor was significantly higher than that produced in the naked pCMV-Luc-injected mice. In particular, the luciferase activity in tumor

tissue after intravenous injection of MPEG-PCL-SS-Tat/pCMV-Luc complex was significantly higher than that in the other normal tissues. The MPEG-PCL-SS-Tat/pCMV-Luc (10/1 weight ratio) complex decreased to a particle diameter of about 60 nm and a zeta potential of about +10 mV, which was sufficient to allow passive delivery into tumor tissue using the EPR effect as described in previous reports (Matsumura and Maeda, 1986). These results strongly suggest that MPEG-PCL-SS-Tat is able to promote the specific delivery of pCMV-Luc into the tumor and that this vector might be useful for delivery of therapeutic genes in the form of pDNA and siRNA and as a non-viral systemic gene carrier targeting tumors for clinical use.

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