Transactivating-transduction protein-polyethylene glycol modified liposomes traverse the blood-spinal cord and blood-brain barriers☆

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Abstract

Naive liposomes can cross the blood-brain barrier and blood-spinal cord barrier in small amounts. Liposomes modified by a transactivating-transduction protein can deliver antibiotics for the treatment of acute bacterial infection-induced brain inflammation. Liposomes conjugated with polyethylene glycol have the capability of long-term circulation. In this study we prepared transactivating-transduction protein-polyethylene glycol-modified liposomes labeled with fluorescein isothiocyanate. Thus, liposomes were characterized by transmembrane, long-term circulation and fluorescence tracing. Uptake, cytotoxicity, and the ability of traversing blood-spinal cord and blood-brain barriers were observed following coculture with human breast adenocarcinoma cells (MCF-7). Results demonstrated that the liposomes had good biocompatibility, and low cytotoxicity when cocultured with human breast adenocarcinoma cells. Liposomes could traverse cell membranes and entered the central nervous system and neurocytes through the blood-spinal cord and blood-brain barriers of rats *via* the systemic circulation. These results verified that fluorescein isothiocyanate-modified transactivating-transduction protein-polyethylene glycol liposomes have the ability to traverse the blood-spinal cord and blood-brain barriers.

Key Words

liposomes; transactivating-transduction protein; polyethylene glycol; blood-spinal cord barrier; blood-brain barrier; caudal vein; fluorescein isothiocyanate; rat; human breast adenocarcinoma cells (MCF-7); cytobiology; neural regeneration

Research Highlights

(1) Transactivating-transduction protein-polyethylene glycol-modified liposomes have the ability of transmembrane and long-term circulation.
(2) Following coculture, cell uptake ability and cytotoxicity were evaluated.
(3) The ability of traversing blood-spinal cord and blood-brain barriers was observed following intravenous injection.
(4) Transactivating-transduction protein-modified liposomes carry drugs and treat intracranial infection. Stability was increased by modification of polyethylene glycol.
(5) Fluorescein isothiocyanate-labeled transactivating-transduction protein-polyethylene glycol-modified liposomes can traverse the blood-spinal cord and blood-brain barrier of rats.

Abbreviations

TAT, transactivating-transduction protein; PEG, polyethylene glycol; FITC, fluorescein isothiocyanate

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INTRODUCTION

Curing central nervous system diseases represents an important challenge for scientists. Pharmaceutical therapy remains the most reliable method for treatment of these diseases[1-2]. However, the limited ability of macromolecular drugs to cross the blood-spinal cord and blood-brain barriers constitutes one of the main problem in this area of medicine. Macromolecular drugs are excluded from the central nervous system by the presence of endothelial cell tight junctions at the blood-spinal cord and blood-brain barriers. Numerous studies have reported interesting approaches to identify a better way to enable drug delivery to the central nervous system through crossing the blood-spinal cord and blood-brain barriers[3-8]. Nanocarriers have been used to increase the stability of administered therapeutic molecules, improve their efficacy, and decrease undesired side effects[9]. Liposomes are well-investigated drug carriers. The plasma membrane presents a formidable barrier to the introduction of macromolecules into cells. Liposomes can diffuse through the endothelial cell membrane and enter the central nervous system passively. Nevertheless, some studies have demonstrated that naive liposomes cannot cross the blood-spinal cord barrier in large amounts to achieve a positive partition coefficient with the concentration administered[10-11]. Transactivating-transduction protein (TAT) is an 11 amino acid portion of the HIV-1 TAT protein that activates transcription of the viral genome[12]. Because traversal through cellular membranes represents a major barrier for efficient delivery of macromolecules into cells, the TAT protein may serve to transport various drugs. It has been widely shown[13-14] that addition of TAT and polyethylene glycol (PEG) to the surface of liposomes enhances their ability to be delivered across cellular membranes and also cross the blood-brain barrier. Few studies have focused on the mechanism by which TAT-PEG-modified liposomes cross the blood-spinal cord and blood-brain barriers. Fluorescence microscopy is among the most important tools to follow the specific interactions of targeted liposomes with cells and elucidate fine details of their intracellular fate[15].

Therefore, this study was designed to analyze the delivery of a novel liposome, as well as its ability to deliver drugs and other molecules across the blood-spinal cord and blood-brain barriers. These novel liposomes have a hydrophobic core for highly effective drug encapsulation and a hydrophilic coat conjugated with PEG and TAT to promote long-term circulation and improved uptake by the central nervous system. Fluorescein isothiocyanate (FITC) was loaded into the liposomes for localization analyses in human breast adenocarcinoma cells (MCF-7), spinal cord tissue and brain.

RESULTS

Quantitative analysis of experimental animals
A total of 30 rats were equally and randomly assigned to three groups and either injected, *via* the caudal vein, with FITC-loaded TAT-PEG-modified liposomes, non-TAT-PEG-modified liposomes and FITC suspension as a control. No rats died during the experiment. A total of 30 rats were included in the final analysis.

Properties of prepared liposomes
The size and shape of the polymeric vesicles were directly observed by transmission electron microscopy. FITC-loaded TAT-PEG-modified liposomes prepared by reverse-phase evaporation at a weight ratio of 2/1 (octadecyl quaternized carboxymethyl chitosan/Chol) are shown in Figure 1.
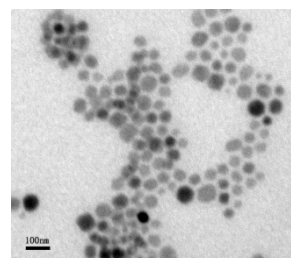

Figure 1 Fluorescein isothiocyanate-loaded liposomes modified by transactivating-transduction protein and polyethylene glycol under transmission electron microscopy (scale bar: 100 μm).

The size of the vesicle is less than 100 nm.

The liposomes were spherical in nature, and the size of the liposomes increased slightly after FITC loading. The vesicles were of different sizes, as could also be seen from the particle size analyzer (Figure 2A). The effective diameter of the polymeric liposomes was 85.9 nm, and their size was slightly smaller than the results obtained from light scattering, possibly because of shrinking of the liposome shell during vacuum drying as part of the sample preparation for transmission electron microscopy. The liposomes had a similar size distribution, characterized by two populations. The predominant population had a size ranging from 80 to 100 nm. By contrast, the zeta potential of FITC-loaded liposomes was 29.57 ± 3.47 mV (Figure 2B), indicating a more stable characteristic.
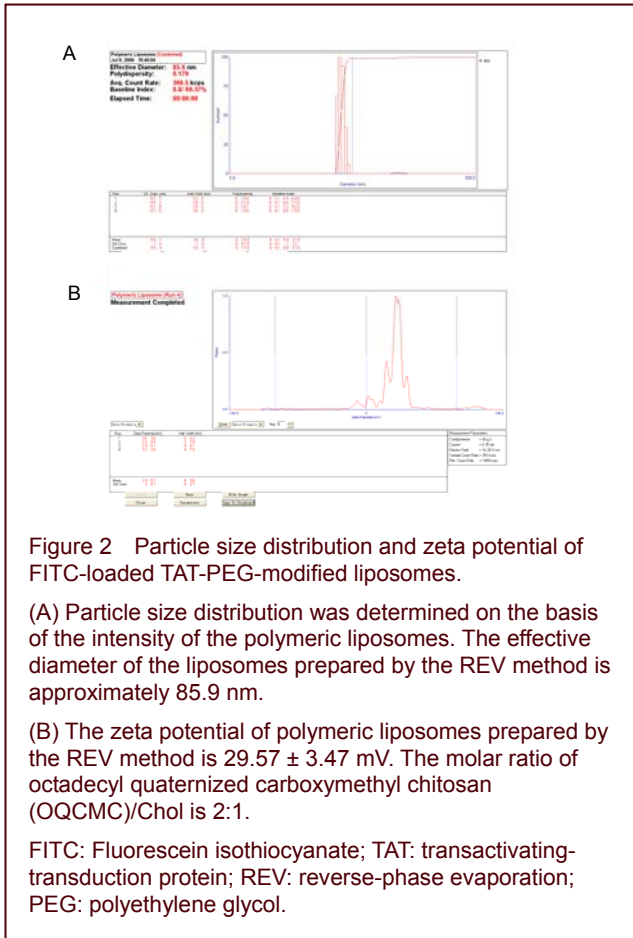
Figure 2   Particle size distribution and zeta potential of FITC-loaded TAT-PEG-modified liposomes.

(A) Particle size distribution was determined on the basis of the intensity of the polymeric liposomes. The effective diameter of the liposomes prepared by the REV method is approximately 85.9 nm.

(B) The zeta potential of polymeric liposomes prepared by the REV method is 29.57 ± 3.47 mV. The molar ratio of octadecyl quaternized carboxymethyl chitosan (OQCMC)/Chol is 2:1.

FITC: Fluorescein isothiocyanate; TAT: transactivating-transduction protein; REV: reverse-phase evaporation; PEG: polyethylene glycol.

Analysis of cell cytotoxicity following coculture of liposomes and MCF-7 cells

After 4, 12, 24 and 48 hours of incubation of liposomes with MCF-7 cells, viability was determined (Figure 3A and Table 1). Non-TAT-modified liposomes exhibited higher cytotoxicity (Figure 3B and Table 1) compared with TAT-PEG-modified liposomes at the same concentration. Whereas cell viability upon incubation with the TAT- PEG-modified liposomes was 61.84 ± 3.34%, cell viability with non-TAT-loaded liposomes was less than 50% at a concentration of 600 µg/mL after 48 hours. Cell viability gradually decreased in proportion to liposome concentration.
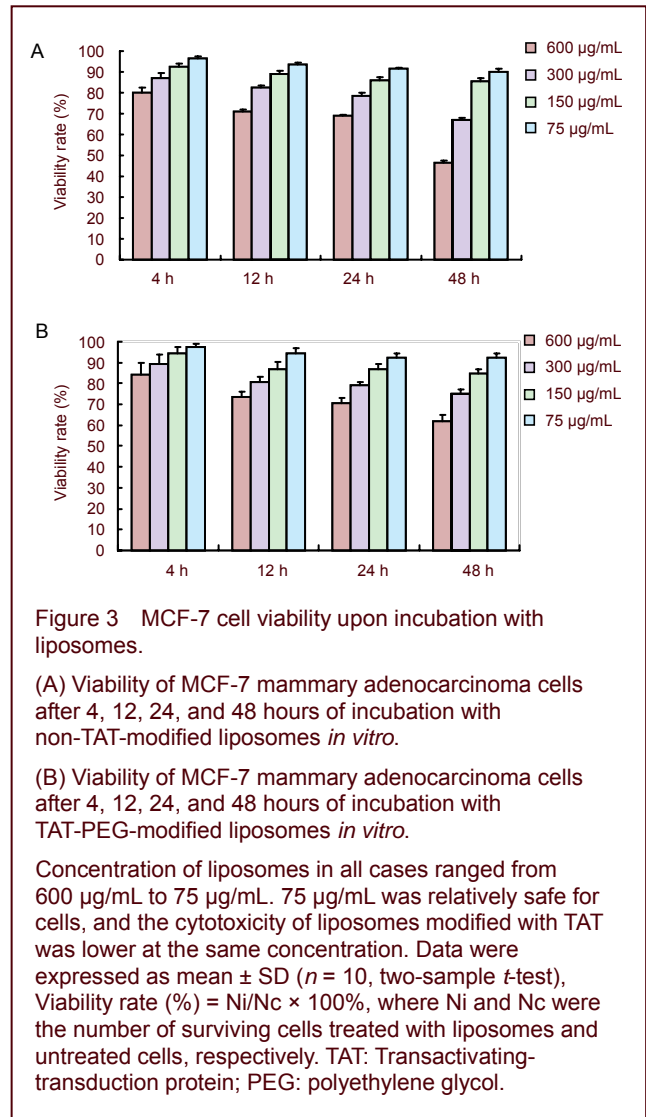

Figure 3   MCF-7 cell viability upon incubation with liposomes.

(A) Viability of MCF-7 mammary adenocarcinoma cells after 4, 12, 24, and 48 hours of incubation with non-TAT-modified liposomes *in vitro*.

(B) Viability of MCF-7 mammary adenocarcinoma cells after 4, 12, 24, and 48 hours of incubation with TAT-PEG-modified liposomes *in vitro*.

Concentration of liposomes in all cases ranged from 600 µg/mL to 75 µg/mL. 75 µg/mL was relatively safe for cells, and the cytotoxicity of liposomes modified with TAT was lower at the same concentration. Data were expressed as mean ± SD (n = 10, two-sample t-test), Viability rate (%) = Ni/Nc × 100%, where Ni and Nc were the number of surviving cells treated with liposomes and untreated cells, respectively. TAT: Transactivating-transduction protein; PEG: polyethylene glycol.
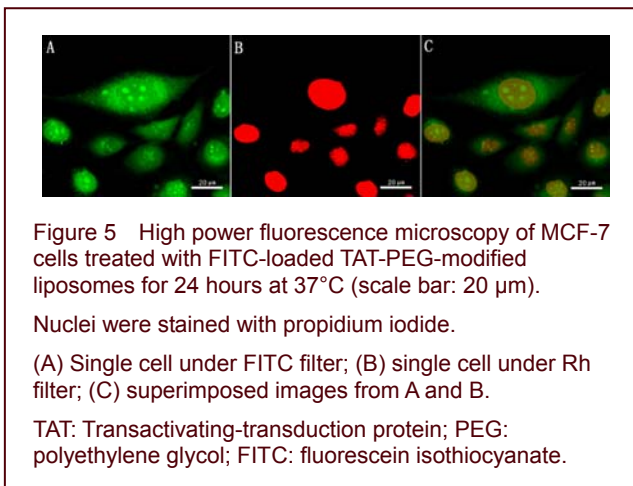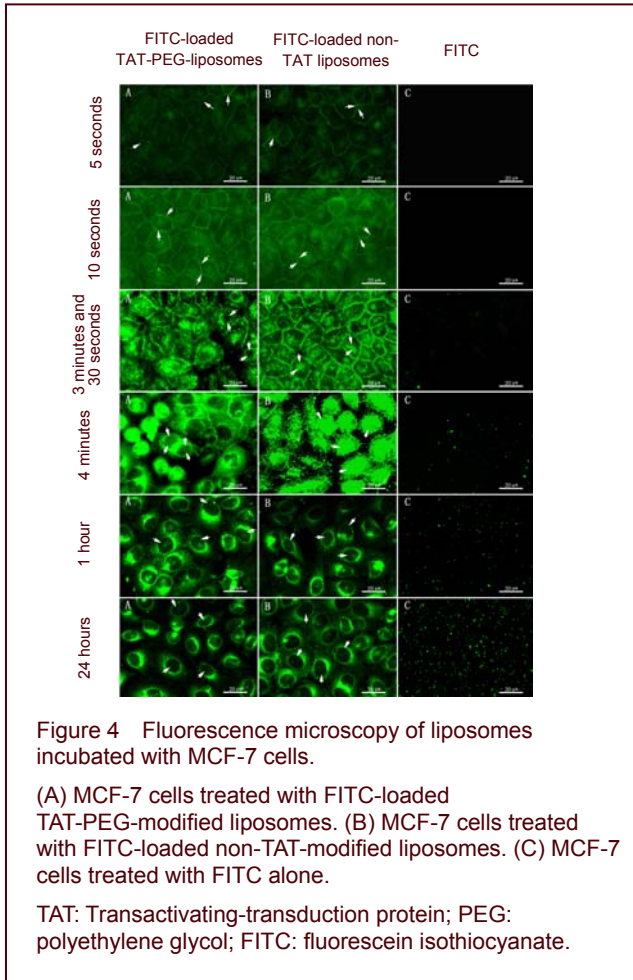
Analysis of liposome uptake by MCF-7 cells *in vitro*

Intracellular localization and liposome fate were investigated in MCF-7 cells using FITC fluorescent label and fluorescence microscopy. Intense intracellular fluorescence was observed after incubation of MCF-7 cells with FITC-loaded TAT-PEG-liposomes. Typical time-dependent distribution patterns of TAT-PEG-modified liposomes are shown in Figures 4 and 5.

Table 1   Viability of MCF-7 cells at different time points with different concentrations of liposomes

Time of coculture (hour)	600 µg/mL		300 µg/mL		150 µg/mL		75 µg/mL	
	non-TAT	TAT	non-TAT	TAT	non-TAT	TAT	non-TAT	TAT
4	80.1±2.5	84.4±5.4[a]	87.1±2.2	89.5±4.3	92.5±1.4	94.4±3.2	96.4±1.0	97.3±1.9
12	71.2±1.1	73.5±2.7[a]	82.4±1.0	80.9±2.4	89.0±1.6	87.0±3.5	93.5±1.2	94.2±2.9
24	68.9±0.7	70.8±2.2[a]	78.5±1.5	79.0±1.8	86.1±1.5	86.8±2.3	91.4±0.8	92.3±2.1
48	46.3±1.4	61.8±3.3[b]	67.1±0.9	75.3±1.8[b]	82.5±1.7	84.7±2.2[a]	90.2±1.3	92.3±2.0[a]

A significant difference between the two groups is apparent after 48 hours of incubation or with a concentration of 600 µg/mL. [a]P < 0.05, [b]P < 0.01, *vs*. non-TAT group. Viability rate (%) = Ni/Nc × 100%, where Ni and Nc were the number of surviving cells treated with liposomes and untreated cells, respectively. Data were expressed as mean ± SD. TAT: Transactivating-transduction protein.

Figure 4   Fluorescence microscopy of liposomes incubated with MCF-7 cells.

(A) MCF-7 cells treated with FITC-loaded TAT-PEG-modified liposomes. (B) MCF-7 cells treated with FITC-loaded non-TAT-modified liposomes. (C) MCF-7 cells treated with FITC alone.

TAT: Transactivating-transduction protein; PEG: polyethylene glycol; FITC: fluorescein isothiocyanate.

Figure 5   High power fluorescence microscopy of MCF-7 cells treated with FITC-loaded TAT-PEG-modified liposomes for 24 hours at 37°C (scale bar: 20 μm).

Nuclei were stained with propidium iodide.

(A) Single cell under FITC filter; (B) single cell under Rh filter; (C) superimposed images from A and B.

TAT: Transactivating-transduction protein; PEG: polyethylene glycol; FITC: fluorescein isothiocyanate.

After 5 seconds, liposome-associated fluorescence appeared around the cellular membrane in the non-TAT- and TAT-PEG-modified liposome groups. The majority of the liposome-associated fluorescence was localized at the cell surface at the midpoint of the cell, indicating close proximity to the surface of the cell membrane. After 10 seconds, the fluorescence was more obvious; however, it remained on the cellular membrane rather than inside the cell. Fluorescence within the cell membrane and cytoplasm in TAT-PEG-modified liposome groups was observed after

3 minutes and 30 seconds. After 4 minutes, intracellular fluorescence was detected; however, it was outside the nucleus. Compared with the TAT-PEG-modified liposome group at the same time points, the fluorescence of non-TAT-PEG-modified liposomes remained outside of the cellular membrane until 1 hour. Liposomes with and without TAT peptide entered the cells after 1 hour (from 1 to 24 hours); no differences in fluorescence localization were observed between the two groups.

Accumulation of liposomes in the rat central nervous system
To determine whether liposomes were able to traverse the blood-spinal cord and blood-brain barriers, rat spinal cord and brain sections were observed at different time points after intravenous injection of FITC, FITC-loaded TAT-PEG-modified liposomes, or FITC-loaded non-TAT-modified liposomes.

Fluorescence was more intense around the blood capillary than the other areas within the spinal cord and brain tissue, demonstrating that the liposomes almost permeated into the surrounding tissue from the capillary (Figures 6A1 and B1). In addition, the liposomes mainly accumulated in the gray matter. Few liposomes were located in the white matter (Figures 6C1 and D1), which may be attributed to the increased perfusion in the gray matter. Many small fluorescent particles, demonstrating tissue aggregation of FITC within the central nervous system, were observed on low power (Figures 6C1 and D1), and visualization using high magnification revealed that many of these fluorescent particles were located within neurocytes (Figure 6). The relationship of these fluorescent particles and cells within the central nervous system was highlighted by comparing the hematoxylin-eosin (Figures 6A2–D2) and fluorescence staining patterns. The results described above were obtained at 4 hours after caudal vein administration. Similar results were seen at 1 hour and even 24 hours after administration, partly because of the long circulation characteristics of PEG.

DISCUSSION

Characteristics of TAT-PEG-modified liposomes
The liposome carrier used in this study has numerous functional groups on the surface and therefore may possess favorable characteristics, such as reducing the toxic side-effects of drugs, selective targeting, solubilization of hydrophobic drugs, stable storage, extended circulation in the blood, and lower interactions with the reticuloendothelial cell system. These liposomes

possess a hydrophobic core for drug incorporation and a hydrophilic shell containing PEG and TAT molecules.
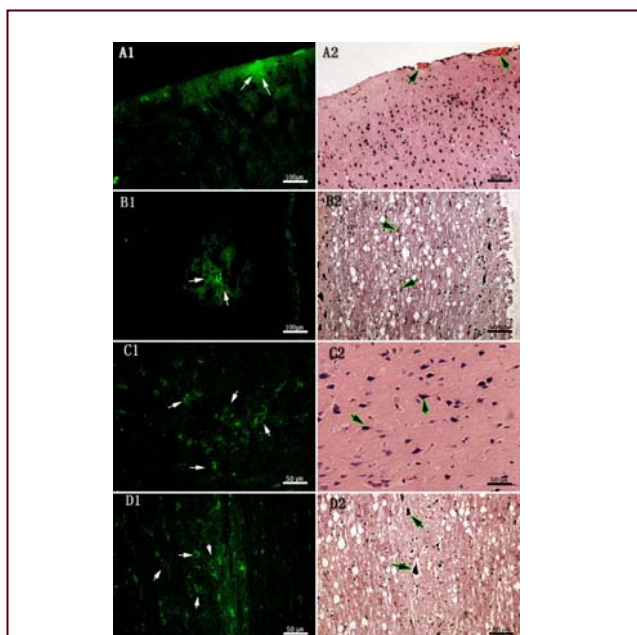

Figure 6 Central nervous system localization of FITC-loaded TAT-PEG-modified liposomes administered intravenously in rats.

Fluorescence (left) and bright-field (right) microscopy of frozen sections were obtained from the spinal cord and brain tissues of rats administered FITC-loaded TAT-PEG-modified liposomes after 4 hours. Arrows show the fluorescent particles accumulated in the neurocytes in the spinal cord and brain.

(A1, B1, C1, D1) Immunofluorescence; (A2, B2, C2, D2) hematoxylin-eosin staining. Scale bars: 100 μm for A1, A2, B1, B2; 50 μm for C1, C2, D1, D2.

TAT: Transactivating-transduction protein; PEG: polyethylene glycol; FITC: fluorescein isothiocyanate.

These characteristics effectively protect the enclosed bioactive compounds against degradation, enabling them to exhibit prolonged activity in the systemic circulation through avoiding being scavenged by the reticuloendothelial cell system. These features also enhance uptake by the central nervous system.

Cytotoxicity analysis of the liposomes

Liposomes induce cytotoxic effects through electrostatic interactions with negatively charged cell membranes, which are dependent on the number, density, and arrangement of the polymer's cationic charges. Increased charges due to high molecular weights and higher charge density of the polymers could induce damaging metabolic and membrane effects, thereby promoting cell death. However, incubation of cells with TAT-PEG-modified and non-TAT-modified liposomes with low densities revealed high cell viability, suggesting that the novel liposomes were biocompatible, and cell

viabilities were not influenced at a relatively safe dose range (75 μg/mL). These results indicate that these novel liposomes could be used as drug carriers without inducing any significant cytotoxic effects.

Analysis of liposome uptake by MCF-7 cells *in vitro*

The uptake of liposomes with TAT was faster and more efficient than that of liposomes without TAT. The presence of the cell-penetrating peptide, TAT, on the liposome surface, promoted cellular uptake. Fluorescence microscopy analysis clearly confirmed the high efficiency of TAT-PEG-liposome uptake. Therefore, it appears that such liposomes can effectively penetrate into these cells in a sufficient number.

Recent studies have shown that internalization of TAT occurs by way of macropinocytosis[16-18], a specialized form of fluid phase endocytosis wherein actin protrusions (circular ruffles) fold in the cell and uptake the surrounding medium. There may be two types of endocytic uptake: clathrin-mediated, and lipid raft-mediated through the formation of caveolae and a non-clathrin, non-caveolar endocytosis termed macropinocytosis. TAT-mediated internalization is a multistep process that involves three main steps. The first step involving binding to the cell surface is thought to be mediated by ubiquitous glycan chains located on the cell surface. Next, macropinocytosis is stimulated by TAT, which might include binding to a cell surface protein by way of proteoglycans or glycolipids[19-21]. Binding to cell surface polyanionic glycan sugar chains, such as heparin sulfate, may play a critical role in TAT internalization. Binding promotes macropinocytosis of TAT into macropinosomes. The last step constitutes escape from macropinosomes into the cytoplasm, which is dependent on the pH drop within endosomes that, along with other factors, facilitates a perturbation of the membrane by TAT and release into the cytoplasm. This final step is believed to be the rate-limiting step in transduction efficiency.

These results indicate that FITC-loaded TAT-PEG-modified liposomes entered MCF-7 cells more rapidly and efficiently than FITC-loaded liposomes lacking the TAT moiety. Therefore, TAT promoted the cellular uptake of the liposomes. We concluded that FITC-loaded TAT-PEG-modified liposomes effectively bind to the surface of MCF-7 cells and are internalized by the cells.

Accumulation of liposomes within the central nervous system

Fluorescence can be detected both inside and outside

the neurocytes in the central nervous system tissue from 1 to 24 hours after administration, which indicates that these liposomes can not only cross the blood-spinal cord and blood-brain barriers, but also can penetrate through the surrounding tissue and into adjacent cells. This is in accordance with the above results for MCF-7 cocultures. It also indicates that the non-TAT-modified liposomes are able to cross central nervous system barriers to a limited extent. However, free FITC was unable to cross the blood-spinal cord and blood-brain barriers.

Naive liposomes cross the blood-spinal cord barrier by passive diffusion. This mechanism can be restricted by many conditions, such as the diameter of the particle, and its molecular weight. The incorporation of a lipid conjugate of PEG resulted in a polymeric layer around the liposome, which reduced the adhesion of plasma proteins that would otherwise cause rapid recognition of the liposomes by mononuclear phagocytes and improved the circulation time of the injected liposomes by decreasing uptake in the reticuloendothelial cell system[22-24]. TAT, one of the cell-penetrating peptides, induces formation of reverse micelles as an energy-independent process[25] and also induces an endocytic event at the plasma membrane, perhaps *via* a mechanism similar to that of adsorptive mediated transcytosis[26-27]. The adsorptive mediated transcytosis mechanism of endocytosis is less influenced by the microenvironment in the central nervous system. Therefore, we combined these two modifications (PEG and TAT) to maximize the penetration of the liposomes into the central nervous system. Our observation that the liposomes modified with PEG and TAT act more effectively supports this choice of modification.

In summary, liposomes modified with PEG and TAT can cross the blood-spinal cord and blood-brain barriers and aggregate into cells in the central nervous system. The liposome carrier used in this research has numerous functional groups on the surface and possesses favorable characteristics for drug-release, targeting, bio-compatibility, and biodegradation. We believe that this modified liposome may be able to carry specific therapeutic agents or bio-active substances and may be useful as a potential new strategy for the treatment of central nervous system disease.

MATERIALS AND METHODS

Design
A cell biology study.

Time and setting
Experiments were conducted in Tianjin Medical University and College of Materials in Tianjin University in China from June 2008 to June 2010.

Materials
Animals
Thirty adult, male, Wistar rats weighing 220 ± 10 g, aged 8 weeks, were provided by the Radiation Study Institute-Animal Center, Tianjin, China (animal license No. SCXK (Jing) 2009-0004). Animals were housed in a ventilated room (10–15 times per hour) under controlled temperature at 20–23°C and humidity of 50–70%. The luminous intensity of the room, controlled with a 12-hour light/dark cycle, was 200 lx, and the noise was 60 db. Free access to food and water was allowed during the experiment. All experimental protocols were in accordance with *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[28].

Vector
Heterobifunctional H_2N-PEGCOOH (PEG: Mn 5000) was ordered from Nektar (San Carlos, CA, USA). TAT peptide (NH_2-CGRKKRRQRRRK) was purchased from GL Biochem Ltd. (Shanghai, China). Chitosan was supplied by Yuhuan Aoxing Biochemistry Co., Ltd. (Zhejiang, China) and had a deacetylation degree of > 99% and a molecular weight of 5×10^4. Glycidyl octadecyl dimethylammonium chloride and carboxymethyl chitosan were all prepared in our laboratory. All other chemicals were of reagent grade and were used as received.

Methods
Culture of human breast adenocarcinoma cells
Human breast adenocarcinoma, MCF-7, cells were maintained in Dulbecco's modified Eagle's medium (ATCC, Bethesda, Maryland, USA) at 37°C and 5% CO_2. DMEM was supplemented with 10% fetal bovine serum, 1 mM Na-pyruvate, 50 U/mL penicillin, and 50 g/mL streptomycin until 80–90% confluence was reached after cells were passaged every 3 days.

Vesicle preparation of octadecyl quaternized carboxymethyl chitosan
Octadecyl quaternized carboxymethyl chitosan, a novel chitosan derivative, is soluble in both water and organic solvents. The quaternization of carboxymethyl chitosan was performed as follows. Carboxymethyl chitosan (5 g) was dissolved in 100 mL of a mixture of de-ionized water saturated with isopropanol. Glycidyl octadecyl dimethylammonium chloride was added slowly with

different molar ratios to the glucosamine unit. The mixture was next trickled with an aqueous NaOH solution (42%) and incubated at 80°C for 24 hours with stirring before being dialyzed for 4 days against water and finally lyophilized to give octadecyl quaternized carboxymethyl chitosan as a white powder.

Octadecyl quaternized carboxymethyl chitosan (150 mg) was dissolved in 10 mL 0.1 M carbonate-bicarbonate buffer, pH 9.12. FITC (5 mg) was dissolved in 5 mL of the same buffer, and then slowly added to the reaction solution, continuously stirring at room temperature away from light for 24 hours. The reaction solution was dialyzed away from light in deionized water, and the dialysis fluid was detected using a full-wavelength scanning fluorescence microplate reader (Sunnyvale, CA, USA) at 520 nm, until the fluorescent absorption at this wave length was not detected. Octadecyl quaternized carboxymethyl chitosan-FITC samples were also obtained by this method.

Preparation of TAT-PEG-modified liposomes

The novel liposomes were prepared from octadecyl quaternized carboxymethyl chitosan/cholesterol[29]. Octadecyl quaternized carboxymethyl chitosan and cholesterol were dissolved in 4 mL of chloroform at room temperature. Their weight ratio was 2:1 (octadecyl quaternized carboxymethyl chitosan:cholesterol). Deionized water (5 mL) was mixed with this organic solvent after that it was sonicated for 10 minutes using a water bath sonicator and evaporated on a rotary evaporator, forming a gel-like, highly concentrated suspension of naive liposomes. The suspension was kept under vacuum for at least 3 hours to remove trace amounts of the organic solvent. TAT peptide and/or PEG were linked to the surface of the naive liposomes using the cross-linking reagent N-hydroxysuccinimidyl-3-(2-pyridyldithio) propionate. The synthesis and schematic representation of the FITC-loaded TAT-PEG-modified liposomes that could encapsulate specific drugs or bioactive molecules are shown in Figures 7 and 8. The FITC-loaded non-TAT-modified liposomes were prepared using the same method outlined above and served as a control.

Morphologies of the different liposomes were observed *via* transmission electron microscopy at an operating voltage of 200 kV with a JEOL-100CX2 instrument (Olympus, Tokyo, Japan) in bright-field mode and by electron diffraction. Dilute suspensions of liposomes in water were dropped onto a carbon-coated copper grid by negative staining with 2% phosphotungstic acid and then air dried.

The average particle size and size distribution were determined by quasielastic laser light scattering with a Malvern Zetasizer (Malvern Instruments Ltd., Malvern, UK) at 25°C. Each experiment was performed in triplicate. The zeta potential was measured with a Zetasizer (Malvern Instruments Ltd.). Zeta limits ranged from –150 to 150 V. Strobing parameters were set as follows: strobe delay –1.00 ms, on time 200.00 ms, and off time 1.00 ms.
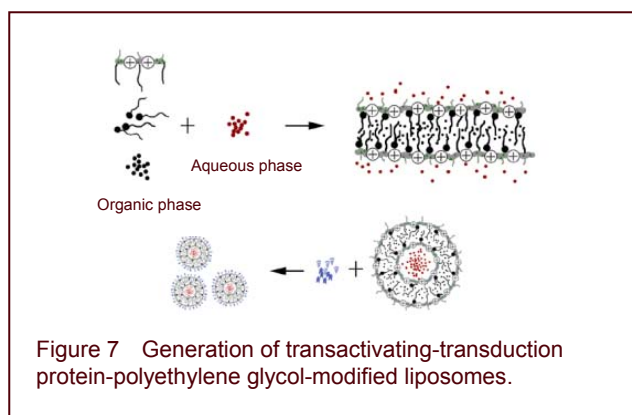

Figure 7　Generation of transactivating-transduction protein-polyethylene glycol-modified liposomes.
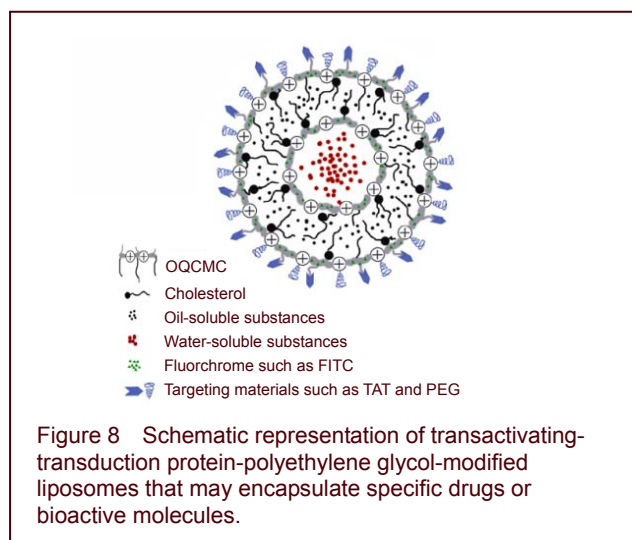

Figure 8　Schematic representation of transactivating-transduction protein-polyethylene glycol-modified liposomes that may encapsulate specific drugs or bioactive molecules.

Cytotoxicity analysis for MCF-7 cells cocultured with liposomes in vitro

The cytotoxicities of TAT-PEG-modified liposomes and non-TAT-modified liposomes were evaluated using the methyl thiazolyl tetrazolium assay[30], and cell viability was determined using cytotoxicity parameters[31]. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin sulfate, and 100 U/mL penicillin at 37°C in humidified 5% CO_2. Cells were seeded at 2 × 10^4 cells/well in a 96-well tissue culture plate, and cytotoxicity testing was performed while cells were in a phase of exponential growth with a doubling time of 48 hours. TAT-PEG-modified liposomes and non-TAT-modified liposomes were applied to the cells at concentrations ranging from 600 to 75 μg/mL. After 4, 12,

24, and 48 hours of incubation, the methyl thiazolyl tetrazolium solution (0.4 mg/mL in PBS) was added to each well and incubated for 4 hours under normal growing conditions. All media was removed, and 150 μL dimethyl sulfoxide was added. After shaking the plate for 30 minutes, absorbance was immediately measured at 570 nm using a microplate reader (EL310, Bio-Tec Instruments Inc., Winooski, VT, USA). Cell viability was expressed as a percentage compared to control cells that had not been treated with liposomes, using the following equation: viability rate (%) = Ni/Nc × 100%, where Ni and Nc were the number of surviving cells treated with liposomes and untreated cells, respectively.

Cellular uptake analysis of liposomes using inverted fluorescence microscopy in vitro

The cell penetration tests of liposomes with and without TAT were performed in MCF7 cells by labeling the liposomes with FITC. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C and 5% CO_2. Cells were seeded onto 6-well plates at a density of 1×10^5 cells per well. After 24 hours, FITC-loaded liposomes with or without TAT and FITC suspension were added to the growth media. After 5 seconds, 10 seconds, 4 minutes, 30 minutes, 1 hour and 24 hours, the medium was removed, and plates were washed with sterile PBS to remove unbound and extracellular substances. Following washing, individual cover slips were mounted cell-side down onto fresh glass slides using a fluorescence mounting medium. Cells were immediately viewed with a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan). Cells were also stained with propidium iodide in PBS (20 μg/mL) for nuclear visualization.

In vivo analysis: rat grouping, caudal vein injection and specimen preparation

Wistar rats were injected with liposome suspension *via* the caudal vein. The rats in the TAT-PEG-modified liposome group were injected with the FITC-loaded TAT-PEG-modified liposomes at a dose of Fe 4.55 mg/kg. Simultaneously, rats in the non-TAT-PEG-modified liposome group were injected with the FITC-loaded non-TAT-modified liposomes, while the rats in control group were injected with an equivalent dose of FITC suspension. After 1, 4, 8, 12, and 24 hours following injection, rats were sacrificed with an intraperitoneal injection of 10% chloral hydrate, and their brains and spinal cords were removed and quickly stored in liquid nitrogen. The tissues were subsequently stored at –80°C for 24 hours and then at –20°C.

Histological analysis for liposome uptake in the central nervous system

Frozen 10 mm coronal sections of the cerebrum and anteroposterior axes sections of the spinal cord were cut and mounted on slides using a fluorescence mounting medium containing DAPI for nuclear staining. Simultaneously, 5 mm-thick frozen sections were cut for hematoxylin-eosin staining. The specimens were observed with a fluorescence microscope (Nikon Eclipse E400, Nikon) and light microscope (JEM 1200EX; Jeol, Tokyo, Japan).

Statistical analysis

All statistical procedures were performed with SPSS 13.0 software (SPSS, Chicago, IL, USA). All data were presented as mean ± SD. The results were analyzed by two-sample *t*-test. A *P*-value less than 0.05 was considered statistically significant.

REFERENCES

[1]   Borisoff JF, Chan CC, Hiebert GW, et al. Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. J Mol Cell Neurosci. 2003;22(3):405-416.

[2]   Kaptanoglu E, Caner H, Solaroglu I, et al. Mexiletine treatment induced inhibition of caspase-3 activation and improvement of behavioral recovery after spinal cord injury. J Neurosurg Spine. 2005;3(1):53-56.

[3]   Ibarra A, Hauben E, Butovsky O, et al. The therapeutic window after spinal cord injury can accommodate T cell-based vaccination and methylprednisolone in rats. Eur J Neurosci. 2004;19(11):2984-2990.

[4]   Akgun S, Tekeli A, Kurtkaya O, et al. Neuroprotective effects of FK-506, l-carnitine and azathioprine on spinal cord ischemia-reperfusion injury. Eur J Cardiothorac Surg. 2004;25(1):105-110.

[5]   Laverty PH, Leskovar A, Breur GJ, et al. A preliminary study of intravenous surfactants in paraplegic dogs: polymer therapy in canine clinical SCI. J Neurotrauma. 2004;21(12):1767-1777.

[6]   Lopez-Vales R, Garcia-Alias G, Fores J, et al. FK 506 reduces tissue damage and prevents functional deficit after spinal cord injury in the rat. J Neurosci Res. 2005; 81(6):827-836.

[7]   Guizar-Sahagun G, Ibarra A, Espitia A, et al. Glutathione monoethyl ester improves functional recovery, enhances neuron survival, and stabilizes spinal cord blood flow after spinal cord injury in rats. J Neurosci. 2005;130(3):639-649.

[8]   Shibuya S, Miyamoto O, Janjua NA. Post-traumatic moderate systemic hypothermia reduces TUNEL positive cells following spinal cord injury in rat. Spinal Cord. 2004; 42(1):29-34.

[9]   Torchilin VP. Cell penetrating peptide-modified pharmaceutical nanocarriers for intracellular drug and gene delivery. Biopolymers. 2008;90(5):604-610.

[10]   Levin VA. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. J Med Chem. 1980;23(6):682-684.

[11]   Begley DJ. Efflux mechanisms in the central nervous system: a powerful influence on drug distribution within the brain. San Diego: Elsevier. 2004.

[12]   Vivès E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem. 1997;272(25):16010-16017.

[13]   Liu L, Venkatraman SS, Yang YY, et al. Polymeric micelles anchored with TAT for delivery of antibiotics across the blood–brain barrier. Biopolymers. 2008;90(5):617-623.

[14]   Liu L, Guo K, Lu J, et al. Biologically active core/shell nanoparticles self-assembled from cholesterol-terminated PEG-TAT for drug delivery across the blood-brain barrier. Biomaterials. 2008;29(10):1509-1517.

[15]   Torchilin VP. Fluorescence microscopy to follow the targeting of liposomes and micelles to cells and their intracellular fate. Adv Drug Deliv Rev. 2005;57(1):95-109.

[16]   Torchilin VP. TAT peptide-mediated intracellular delivery of pharmaceutical nanocarriers. Adv Drug Deliv Rev. 2008; 60(4-5):548-558.

[17]   Gupta B, Torchilin VP. Transactivating transcriptional activator-mediated drug delivery. Expert Opin Drug Deliv. 2006;3(2):177-190.

[18]   Murriel CL, Dowdy SF. Influence of protein transduction domains on intracellular delivery of macromolecules. Expert Opin Drug Deliv. 2006;3(6):739-746.

[19]   Gump JM, Dowdy SF. TAT transduction: the molecular mechanism and therapeutic prospects. Trends Mol Med. 2007;13(10):443-448.

[20]   Amand HL, Fant K, Nordén B, et al. Stimulated endocytosis in penetratin uptake: effect of arginine and lysine. Biochem Biophys Res Commun. 2008;371(4): 621-625.

[21]   Metselaar JM, Bruin P, de Boer LW, et al. A novel family of L-amino acid-based biodegradable polymer-lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. Bioconjugate Chem. 2003;14(6):1156-1164.

[22]   Bazile DV, Ropert C, Huve P, et al. Body distribution of fully biodegradable [14C]-poly(lactic acid) nanoparticles coated with albumin after parenteral administration to rats. Biomaterials. 1992;13(15):1093-1102.

[23]   Landry FB, Bazile DV, Spenlehauer G, et al. Influence of coating agents on the degradation of poly (lactic acid)nanoparticles in model digestive fluids (USP XX2). STP Pharm Sci. 1996;63:195-202.

[24]   Torchilin VP, Papisov MI. Hip´otesis: Why do polyethylene glycol-coated liposomes circulate so long? J Liposome Res. 1994;4:725-739.

[25]   Torchilin VP, Rammohan R, Weissig V, et al. TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proc Natl Acad Sci U S A. 2000;98(15):8786-8791.

[26]   Richard JP, Melikov K, Vives E, et al. Cell-penetrating peptides: a reevaluation of the mechanism of cellular uptake. J Biol Chem. 2003;278(1):585-590.

[27]   Green I, Christison R, Voyce CJ, et al. Protein transduction domains: are they delivering? Trends in Pharm Sci. 2003;24(5):213-215.

[28]   The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

[29]   Liang XF, Wang HJ, Luo H, et al. Synthesis, structure, and properties of novel quaternized carboxymethyl chitosan with drug loading capacity. Wuli Huaxue Xuebao. 2008;24: 223-229.

[30]   Kim SY, Lee YM, Shin HJ, et al. Indomethacin loaded methoxy poly (ethylene glycol)/poly(e-caprolactone) diblock copolymeric nanosphere: pharmacokinetic characteristics of indomethacin in the normal sprague-dawley rats. Biomaterials. 2001;22(14):2049-2056.

[31]   Espuelas S, Legrand P, Irache JM, et al. Poly (e-caprolactone) nanospheres as an alternative way to reduce amphotericin B toxicity. Int J Pharm. 1997;158: 19-27.

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