

A peptide-mediated targeting gene delivery system for malignant glioma cells

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Abstract: Glioblastoma multiforme (GBM) is the most common and malignant glioma. Although there has been considerable progress in treatment strategies, the prognosis of many patients with GBM remains poor. In this work, polyethylenimine (PEI) and the VTWTPQAWFQWV (VTW) peptide were modified and synthesized into GBM-targeting nanoparticles. The transfection efficiency of U-87 (human glioblastoma) cells was evaluated using fluorescence microscopy and flow cytometry. Cell internalization was investigated to verify the nanoparticle delivery into the cytoplasm. Results showed that the methods of polymer conjugation and the amount of VTW peptide were important factors to polymer synthesis and transfection. The PEI-VTW₂₀ nanoparticles increased the transfection efficiency significantly. This report describes the use of VTW peptide-based PEI nanoparticles for intracellular gene delivery in a GBM cell-specific manner.

Keywords: glioblastoma, polyethylenimine, nanoparticles, drug-delivery systems, gene transfer techniques

Introduction

Glioblastoma multiforme (GBM) is a severely malignant glioma, which accounts for approximately 1/5–1/4 of the primary intracranial malignancies.^{1,2} Despite the progress that has been made in cancer treatment, the prognosis for a portion of patients with GBM remains poor, with a median survival of approximately 3–15 months.^{1,3–5} Over the past few decades, gene therapies, including the use of suicide genes^{6–8} and small interfering RNA (siRNA)^{9,10} have demonstrated potential as cancer treatments. However, one obstacle to gene therapy is the lack of an effective, safe, and targeting vector.^{6,7} Viral vectors have higher transfection efficiency than nonviral ones, but they also have immunogenicity and potential oncogenicity implications.^{6,7} Nonviral vectors made of polymers, such as polyethylenimine (PEI), poly-L-lysine (PLL), and chitosan, have been used increasingly widely because of their biosafety, convenience of preparation, and stability.^{11–13} PEI is considered one of the good nonviral vectors, and it shows higher transfection efficiency than PLL and chitosan.^{14–16} However, the PEI/DNA nanoparticles are still less effective than viral vectors and have no targeting effect.^{14,16,17}

Certain molecules overexpressed on the surface of some tumor cells, including fibroblast growth factor receptors and epidermal growth factor receptors, have been used to improve the penetration of drugs or genetic products into tumor cells.^{18–20} However, these molecules are not specific enough because they are also expressed on some normal cells, although not to the same extent as on tumor

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cells. Phage peptide display is one technique that can be used to isolate peptides with targeting effects on specific cells.²¹ One peptide recently found by phage display, with a sequence of VTWTPQAWFQWV (VTW), showed particularly strong binding to human glioblastoma cell lines, but quite weak binding to normal human astrocytes and other cell lines.²²

In this study, we constructed PEI/DNA nanoparticles conjugated with VTW to investigate whether these nanoparticles could specifically increase the transfection efficiency of malignant glioblastoma cells.

Materials and methods

Materials

Branched PEI (25 kDa), N,N-Dimethylformamide (DMF), PLL (70–150 kDa) and trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The PD-10 desalting columns were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Mashikimachi, Kamimashiki-gun Kumamoto, Japan). The VTW peptide with a cysteine residue at the C-terminus, with a purity of >95%, was purchased from Science Peptide Biological Technology Co, LTD (Shanghai, People's Republic of China). The peptide was stored in small aliquots at -80°C . The fluorescein isothiocyanate (FITC) nucleic acid labeling kit was purchased from Mirus Bio (Madison, WI, USA). Antibiotics, L-glutamine, 0.25% trypsin, fetal bovine serum (FBS), minimum essential medium (MEM), Neuralbasal[®] medium, 2.5S nerve growth factor, B27 supplement, Lipofectamine[®], and phosphate-buffered saline (PBS) were purchased from Invitrogen Life Technologies, (Carlsbad, CA, USA). Other chemical reagents were commercially available and of analytical grade.

Cell culture

The U-87 (human glioblastoma) cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China). The T98G (human glioblastoma), U251 (human glioma), A549 (human lung adenocarcinoma), and PC12 (rat adrenal gland pheochromocytoma) cell lines were gifts from the Key Laboratory of Qilu Hospital of Shandong University, Jinan, People's Republic of China. All cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin–streptomycin.

The neural cells from rat cerebral cortex were obtained and cultured as previously described, with slight differences.²³ The animal experiments complied with the experimental animal ethical approval and inspection of the Research Subject Review Board at Shandong University, People's Republic of China. The 1-day-old neonatal Sprague Dawley (SD) rats were obtained from the Lab Animal Center of Shandong University, euthanized using the inhalant isoflurane, and then decapitated. After the cranium and meninges were removed, the cerebral cortex was dissected in a sterile environment and dissociated with 0.25% trypsin for 10–15 min at 37°C . Cells were seeded in 24-well plates coated with 0.1 mg/mL poly-L-lysine at a density of 2×10^5 cells/well in Neurobasal medium supplemented with 20 ng/mL nerve growth factor, 2% B27 supplement, 2 mM L-glutamine, and 1% penicillin–streptomycin.

Plasmid DNA (pDNA) preparation

The enhanced green fluorescent protein plasmid (pEGFP) was transfected into *Escherichia coli* DH5 α competent cells (Tiangen, Beijing, People's Republic of China) and amplified. DNA purification was performed using an EndoFree Plasmid Kit (Tiangen), following the manufacturer's instructions. The concentration and purity of the plasmid preparation were measured by ultraviolet (UV) spectrophotometer (DU800; Beckman Coulter, Brea, CA, USA). Plasmid solutions with absorbance ratios (260 nm/280 nm) between 1.8 and 2.0 were used.

Synthesis of VTW-modified PEI copolymers

Three types of VTW-modified PEI nanoparticles were made to investigate whether the amount of VTW conjugated with PEI influenced the transfection efficiency. PEI (1 μmol) dissolved in 1 mL hepes-buffered saline (HBS) (0.5 M NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4) was mixed with 100 μL SPDP (100 mM in DMF) and incubated under argon protection for 2 hours at room temperature (RT). This pyridyldithio (PDT)-activated PEI was named PEI-PDT₁₀. For the other two groups (PEI-PDT₂₀ and PEI-PDT₄₀), the same protocol was used, except the volumes of SPDP used were 200 μL and 400 μL , respectively. The PEI-PDT polymers were purified using a PD-10 column, according to the manufacturer's instructions. The VTW peptide was dissolved in 100 μL DMF and 900 μL guanidine hydrochloride (GnHCl) buffer (6 M GnHCl, 0.5 M NaCl, 20 mM HEPES, pH 7.4). Then, the VTW solution with different molar ratios to PEI-PDT (10:1, 20:1, 40:1)

was dropped into three types of PEI-PDT solutions and incubated for 2 hours at RT. The PDT residue of PEI-PDT was displaced by the sulfhydryls of the cysteine residue of VTW, forming the VTW-modified PEI copolymers (PEI-VTW, here abbreviated as PV₁₀, PV₂₀ and PV₄₀). Subsequently, the copolymers were purified separately by using a 30 kDa cut-off filter (Vivaspin 2; Sartorius, Göttingen, Germany) and centrifugation at 12,000 Xg for 20 minutes. The concentrations of PEI-VTW in the samples were measured by TNBS assay as described previously²⁴ and with the pyridine-2-thione assay. The level of SPDP-modification was determined by measuring the absorbance of pyridine-2-thione at 343 nm. All solutions were filtered through 0.22 µm Merck Millipore (Billerica, MA, USA) membranes and stored at -80°C.

Preparation of PV nanoparticles

The PV₁₀, PV₂₀, and PV₄₀ copolymer solutions were separately diluted with hepes-buffered glucose (HBG) (20 mM HEPES, 5% w/v glucose, pH 7.4) and incubated at RT for 5 minutes. Then, the pDNA solution (0.1 mg/mL) was mixed with different copolymer solutions separately by vortexing for 30 seconds and incubated for 30 minutes at RT at ratios of nitrogen in PEI amino residues to phosphates of DNA (N/P ratios) ranging from 1 to 10. The pDNA was condensed with the PV copolymers by electrostatic force and formed into nanoparticles. The PEI nanoparticles were made by the same procedure.

Characterization of PV nanoparticles

Particle size and zeta potential

The particle size and surface charge of nanoparticles in HBG were measured with a Delsa™ Nano (Beckman Coulter) equipped with a standard capillary electrophoresis cell and dynamic light scattering. All the nanoparticles were measured in HBG buffer (pH 7.4, ionic strength 0.02 mol·dm⁻³) at RT. The measurements were performed at least in triplicate.

Transmission electron microscopy (TEM) imaging

One drop of suspension was placed on carbon holey film on a copper TEM grid and stained with 2% phosphotungstic acid solution for 20 seconds. The grid was allowed to dry for 10 minutes and then examined with a JEM-200CX TEM (JEOL, Tokyo, Japan).

Agarose gel electrophoresis assay

The ability to condense plasmid DNA of PEI and PV nanoparticles was examined with agarose gel electrophoresis. Electrophoresis was carried out with 1.0% agarose gels at

100 V for 30 minutes in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer (40 mM Tris-hydrogen chloride, 1 v/v% acetic acid and 1 mM EDTA). The retardation of the nanoparticles was visualized by staining with ethidium bromide and illuminating with UV using a FluorChem™9900 (Cell Biosciences, Heidelberg, VIC, Australia).

Nuclear magnetic resonance (NMR) analysis

The ¹H NMR spectra of copolymers was determined by an AVANCE (300 MHz) NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a 5 mm broadband observe (BBO) probe at RT with a 2-second relaxation delay.

In vitro transfection studies

Fluorescence microscopy

The U-87, T98G, and U251 cells were seeded into 24-well plates at a density of 1 × 10⁵ cells per well and were rinsed with fresh MEM when they grew to 70%–80% confluence. Then, the freshly prepared nanoparticles, with 1.0 µg of pEGFP at an N/P ratio of 10, were added to the cells and incubated in 500 µL serum-free MEM at 37°C for 5 hours. Subsequently, the transfection medium was replaced with complete culture medium, and the cells were incubated for an additional 24 hours. Then, the images were captured with a fluorescence microscope (Olympus BX-51). PEI/DNA nanoparticles were also prepared at an N/P ratio of 10:1. Lipofectamine-DNA complexes were used as positive control and naked DNA as the negative control. Nanoparticles made of PV₂₀ were incubated with A549, PC12, and neural cells under the same transfection conditions. The influence of serum on transfection was examined by adding 10% FBS to the incubation medium in two other groups of PV₂₀ nanoparticles and Lipofectamine. All the experiments were performed at least in triplicate.

Flow cytometry

U-87 cells were seeded at a density of 3 × 10⁵ cells/well in 6-well plates. Transfection procedures were as described above, but the dose of nanoparticles was three times larger. Nanoparticles were delivered to each well in 1.5 mL MEM and incubated for 5 hours. Then, the medium was replaced with fresh culture medium. After 24 hours, the cells were collected, washed twice with PBS, and resuspended in PBS. Flow cytometry was performed with a FACS-Calibur (BD Biosciences, San Jose, CA, USA). Transfection efficiency was then assessed in cell counts of 10,000 per sample by analyzing for EGFP fluorescence using BD CellQuest™ Pro (Version

5.2.1) (BD Biosciences). The assessments were performed at least in triplicate.

Internalization of nanoparticles

The cellular uptake experiment of the nanoparticles loaded with FITC-labeled plasmid was performed to test the internalization ability and lysosomal escape ability of the PV₂₀ nanoparticles. Fluorescent labeling of the pDNA was carried out using Label IT[®] Kits (Mirus Bio) according to the manufacturer's instructions. The labeled DNA was stored at -20°C and protected from light. Lysosomes were marked by Lyso-Tracker Red (Beyotime, Haimen, People's Republic of China) to verify the location of the nanoparticles in the cytoplasm. The transfection of the U-87 cells was carried out according to the procedure described above. After 3 hours' incubation, the cells were washed twice with PBS. Images of the cells were captured with a LSM710-confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Competition assay

Cells were pre-incubated at 37°C for 1 hour with a 1,000 molar excess of free VTW peptide compared with peptide conjugated to the PV₂₀ nanoparticles. Then, the internalization experiment and microscopy with PV₂₀ nanoparticles were performed as described above.

Cytotoxicity assay

Cytotoxicity was determined using a CCK-8 Assay Kit according to the manufacturer's instructions. Transfection procedures were as described above but in 96-well plates. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific). Each evaluation was performed at least in triplicate.

Statistical analysis

Data in this study were calculated as the mean ± standard deviation. One-way analysis of variance (ANOVA) and a Tukey's post hoc test were used. A *P*-value less than 0.05 was considered statistically significant.

Results

Characteristics of nanoparticles

The yield of polymers was 78%–81% according to the TNBS assay. The actual ratio of activated amines in PEI was a little lower than the theoretical ratio (PEI-PDT₁₀, 7.7 ± 0.35; PEI-PDT₂₀, 16.1 ± 1.06; PEI-PDT₄₀, 33.6 ± 1.63) according to the pyridine-2-thione assay. The nanoparticles in all groups were spherical under TEM (Figure 1A–D). Nanoparticle size and

zeta potential were measured to describe the physical features of the nanoparticles at an N/P ratio of 10 (Figure 1E). The PEI/DNA nanoparticle was 69.8 ± 5.71 nm in diameter and the zeta potential was 27.2 ± 1.62 mV. The polydispersity indices of PEI, PV₁₀, PV₂₀, and PV₄₀ nanoparticles were 0.273, 0.165, 0.291, and 0.212, respectively. The PV nanoparticles were larger than the PEI nanoparticles and had lower zeta potential values. In agarose gel electrophoresis, uncomplexed DNA migrated in the lane toward the positive terminal, whereas completely complexed DNA was retained in the loading well. The unmodified PEI formed complete complexes with pDNA at an N/P of 2 (Figure 1Fc), whereas PV₁₀, PV₂₀ and PV₄₀ formed complexes at N/P ratios of 3, 5 and 6 (Figure 1Fd–f). In the 1H NMR spectra (Figure 1G), all the peaks at 2.7–2.2 ppm belonged to chemical shifts of (CH₂CH₂NH)_n in PEI-PDT; the peaks at 5.0 ppm were assigned to -NH₂ and those at 7.1–8.2 were attributed to pyridine. In the spectra of the PV copolymer, there still were signals of -NH₂ and (CH₂CH₂NH)_n and new signals of -CONH-, -CH₂, and -CH₃, indicating the VTW peptide, but pyridine signal disappeared, which meant that conjugation succeeded (Figure 1G).

Transfection efficiency

The scheme of the nanoparticle preparation and transfection of glioma cells is illustrated in Figure 2. U-87 was transfected with pEGFP nanoparticles complexed with PEI and PV separately. In general, with the U-87 cells, all the PV nanoparticles showed higher transfection efficiencies than the PEI nanoparticles, and the PV₂₀ nanoparticles showed the best result (68.59% ± 2.13%) (Figures 3 and 4A). With the T98G and U251 glioma cells, the PV nanoparticles also showed better transfection results than the PEI nanoparticles, although not as good as the transfection results with U-87 cells (Figures 3 and 4B and C). The transfection efficiency of PV₂₀ nanoparticles for the U-87, T98G, and U251 cells was still high, and even higher than with Lipofectamine (for U-87 cells) after adding serum to the incubation medium, although a little lower than without serum (Figure 4A–C). Primary neural cells, A549 and PC12 cells, were transfected with PV₂₀ nanoparticles as the control. As expected, the transfection efficiency of A549 and PC12 cells was not improved by PV₂₀ nanoparticles (Figures 3 and 4D). Furthermore, these nanoparticles did not show any effect on the transfection of primary neural cells from rat cerebral cortex (Figures 3 and 4D).

Cytotoxicity of nanoparticles

The cytotoxicity of PEI and PV nanoparticles was evaluated. All the PV nanoparticles exhibited less toxicity to U-87, T98G,

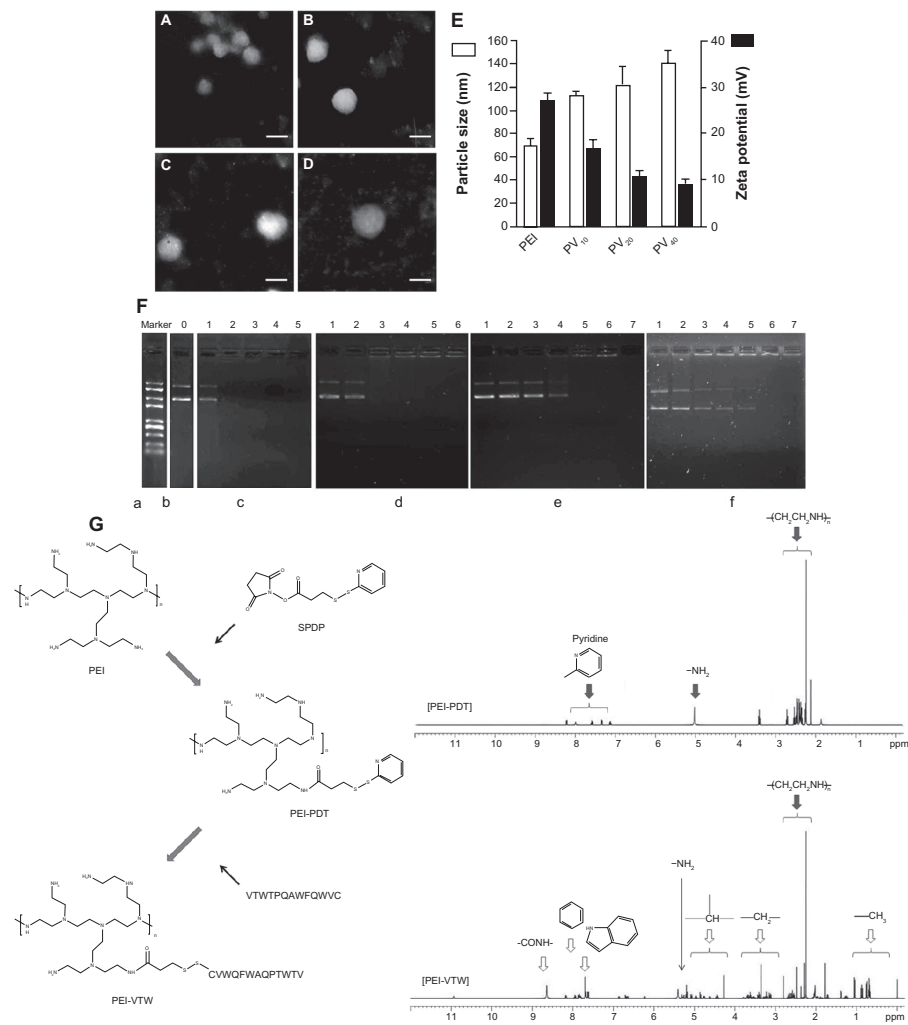


Figure 1 TEM, particle size, zeta potential, agarose gel electrophoresis, and NMR images.

Notes: All nanoparticles, PEI (A), PV₁₀ (B), PV₂₀ (C), and PV₄₀ (D), were made at an N/P of 10 (scale bar: 100 nm). The detailed data of particle size and zeta potential are shown in (E). (F) shows the DNA ladder marker (a) and that PEI complexed DNA completely at an N/P of 2, PV₁₀ at 3, PV₂₀ at 5, and PV₄₀ at 6 (b). The different results from gel electrophoresis of PEI (c), PV₁₀ (d), PV₂₀ (e), and PV₄₀ (f) complexed with pDNA at various N/P ratios are shown. (G) shows the characterization of PV polymer by 1H NMR spectra. The PEI was activated into PEI-PDT by SPDP, and then the PEI-PDT was conjugated with VTW peptide and made into PV.

Abbreviations: NMR, nuclear magnetic resonance; N/P, nitrogen in PEI amino residues to phosphates of DNA; PDT, pyridyldithio; PEI, polyethylenimine; PV, VTW-modified PEI copolymers; SPDP, N-succinimidyl 3-(2-pyridyldithio)-propionate; TEM, transmission electron microscopy; VTW, VTWTPQAWFQWV; pDNA, plasmid DNA.

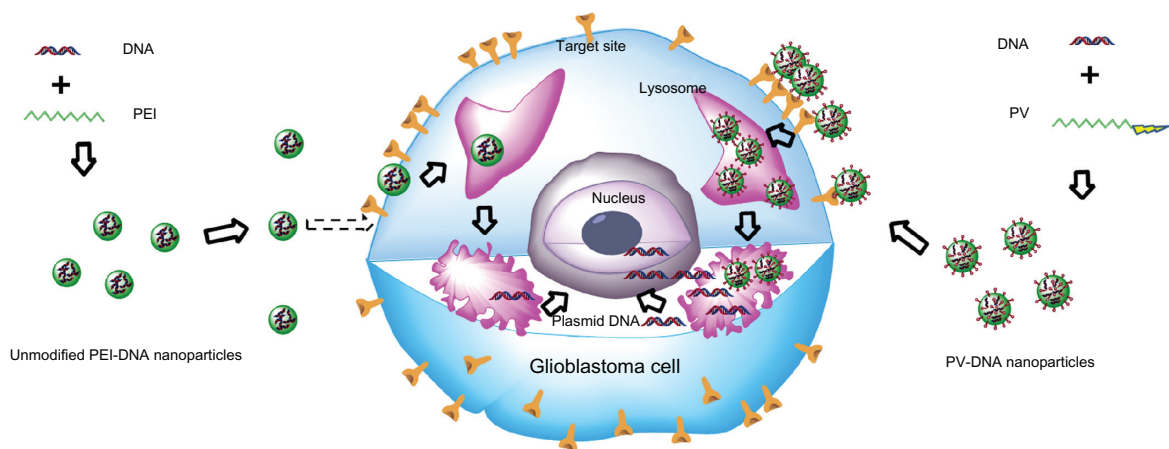


Figure 2 The synthesis of nanoparticles with plasmid DNA made of PEI or PV and their internalization.

Abbreviations: PEI, polyethylenimine; PV, VTW-modified PEI copolymers; VTW, VTWTPQAWFQWV.

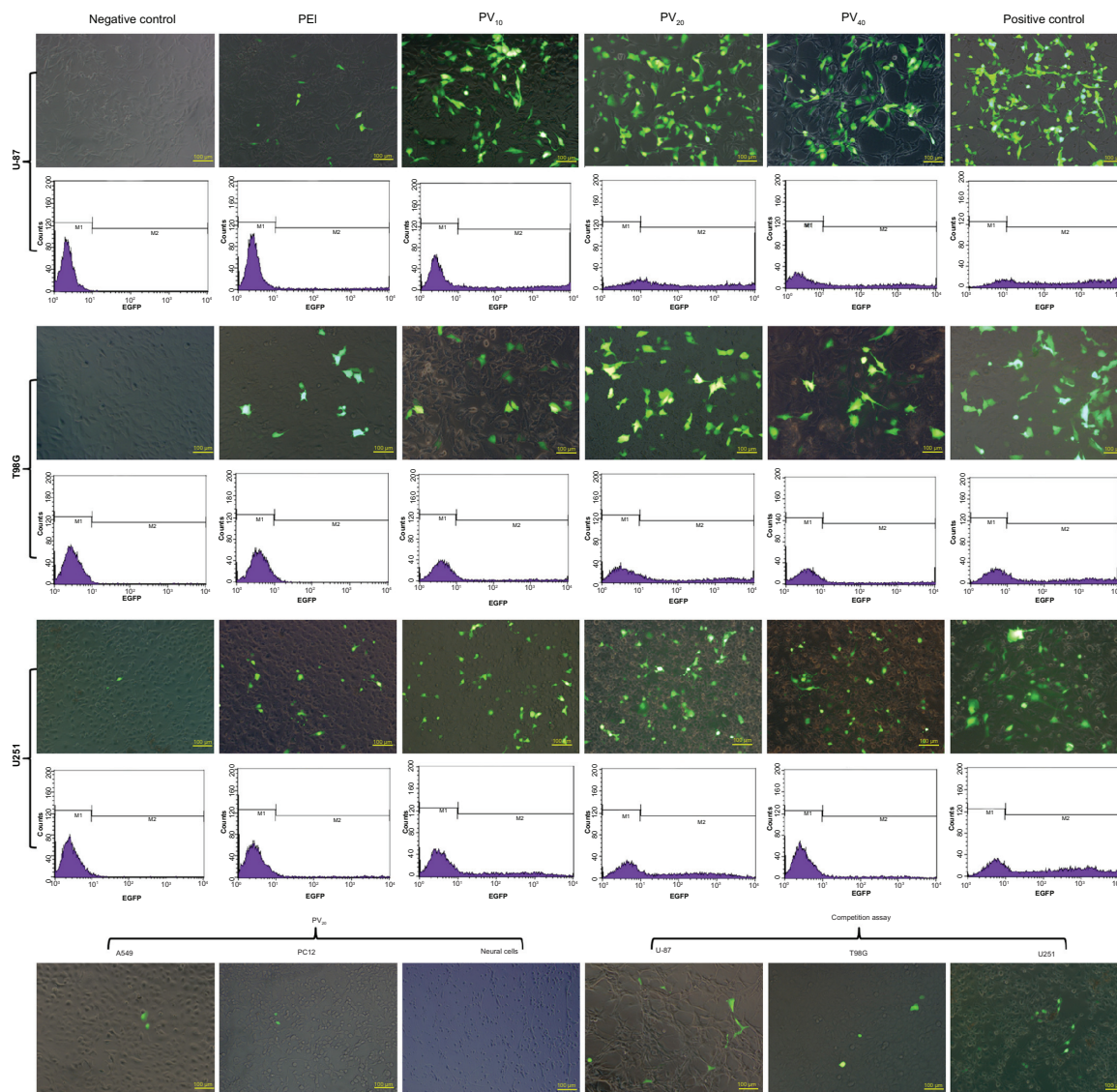


Figure 3 Transfection efficiency of nanoparticles for different kinds of cells.

Notes: Fluorescent micrographs and flow cytometry results of U-87 cells transfected with PV_{10} , PV_{20} , and PV_{40} nanoparticles at the N/P of 10 are presented. The transfection results of T98G and U251 are also shown. The fluorescent images were merged with optical ones. Negative control: naked pDNA; positive control: Lipofectamine® (Life Technologies, Carlsbad, CA, USA)–pDNA complexes. The PV_{20} nanoparticles showed very low transfection effect for PC12 and A549 cells and absolutely no effect for neural cells. Competition assays for glioma cells are shown. Scale bar: 100 μ m.

Abbreviations: N/P, nitrogen in polyethylenimine amino residues to phosphates of DNA; pDNA, plasmid DNA; PEI, polyethylenimine; PV, VTWTPQAWFQWV-modified PEI copolymers.

and U251 cells than the PEI nanoparticles (Figure 4A–C). The PV_{20} nanoparticles also had very low cytotoxicity to neurons compared with the PEI nanoparticles (Figure 4D).

Internalization of nanoparticles

In the cytoplasm of U-87 cells that were incubated with PV_{20} nanoparticles after 1 hour, there was colocalization of lysosomes and PV_{20} nanoparticles (Figure 5). After 4 hours' incubation, there was no obvious colocalization, which demonstrates good cellular uptake and escape from the lysosomes.

Competition assays

In competition assays, there was very low transfection efficiency in U-87, T98G, and U251 cells (Figures 3 and 4A–C) and no distribution of PV_{20} nanoparticles in U-87 cells (Figure 5), which means the uptake of PV_{20} nanoparticles into U-87 cells was nearly completely blocked after the cells were pre-incubated with free VTW peptide.

Discussion

GBM is a highly aggressive brain tumor in human beings, which is difficult to remove completely, prone to recur,

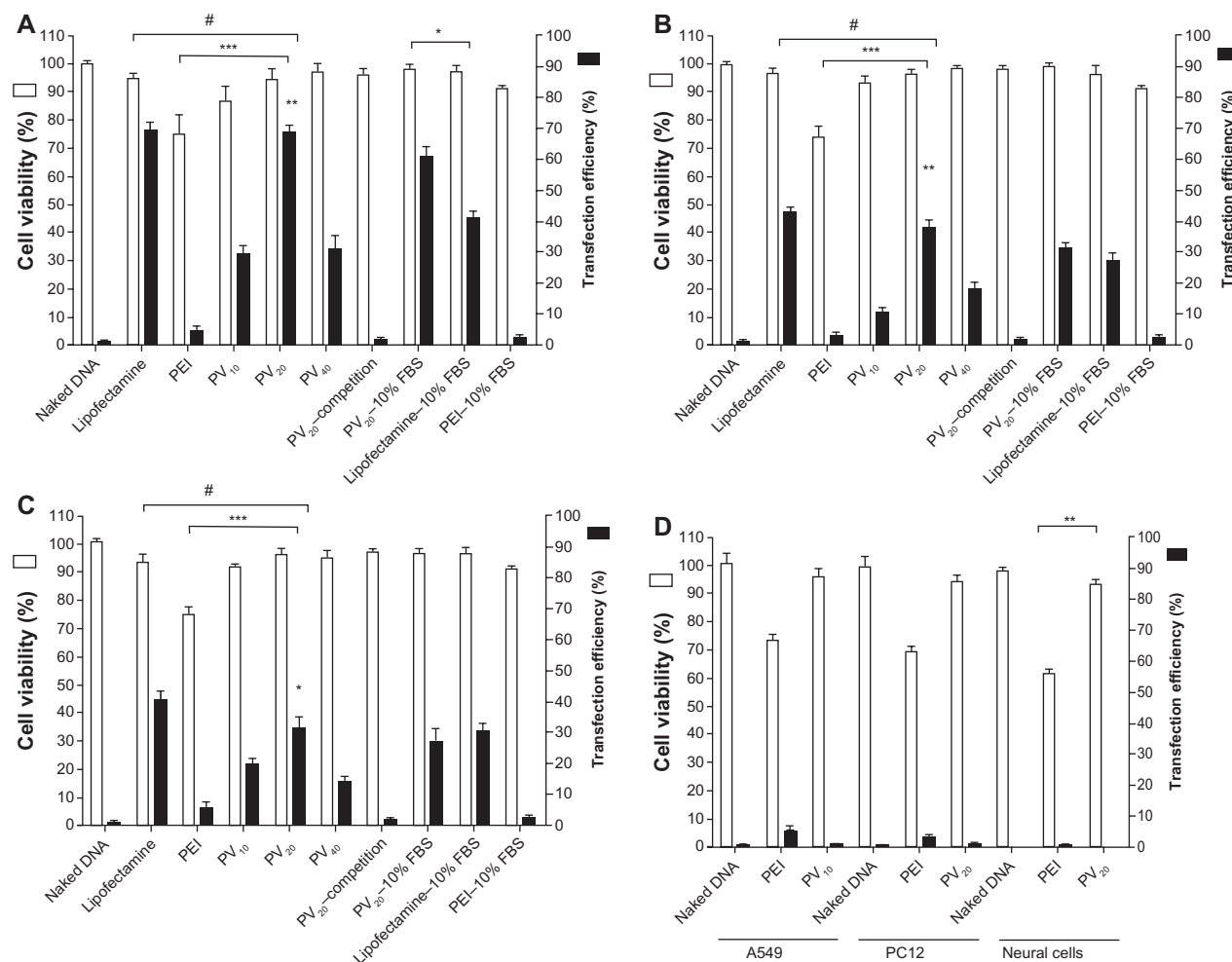


Figure 4 The histogram of the transfection efficiency and cell viability of different cells treated with nanoparticles.

Notes: (A) U-87 cells; (B) T98G cells; (C) U251 cells; (D) A549, PC12, and neural cells. Negative control: naked pDNA; positive control: Lipofectamine® (Life Technologies, Carlsbad, CA)–pDNA complexes. The percent cell viabilities of different nanoparticles are shown. U-87 cells were transfected with all kinds of nanoparticles at the N/P of 10. The transfection efficiency of PV₂₀ nanoparticles was better than the other PEI-based nanoparticles to U-87 and T98G cells (A and B) and U251 cells (C), and especially higher than that of the PEI nanoparticles to U-87 and T98G cells (A and B) and U251 cells (C). The transfection efficiency of PV₂₀ nanoparticles to U-87 and T98G cells was close to that of Lipofectamine–pDNA complexes (A and B), a little lower than that of Lipofectamine–pDNA complexes to U251 cells (C). The PV₂₀ nanoparticles can resist the influence of serum very well, with higher transfection efficiency than Lipofectamine at the same condition (A). The competition assay showed the transfection efficiency to U-87, T98G, and U251 was all decreased significantly after the pre-incubation with free VTW peptide. The PV₂₀ nanoparticles showed very low transfection effect to A549 and PC12 and absolutely no effect to neural cells, meanwhile showing a very low cytotoxicity to neural cells compared with PEI nanoparticles. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; # $P > 0.05$.

Abbreviations: FBS, fetal bovine serum; N/P, nitrogen in polyethylenimine amino residues to phosphates of DNA; pDNA, plasmid DNA; PEI, polyethylenimine; PV, VTW-modified PEI copolymers; VTW, VTWTPQAWFQWV.

and causes severe damage to the brain.^{1,4,5} Although gene therapy has been acknowledged as a promising strategy, there are still several disadvantages,^{3,5} including the need for specifically targeting the cancer cells.²⁵ It is a challenge to target and deliver genetic materials to GBM cells specifically.^{6,7,11,14} It has been reported that many carriers can augment the efficiency of introducing drugs or nucleotides into glioma cells.^{20,26–29} One peptide, called VTW, which can selectively bind to GBM cells, indicates another promising approach.²² One report showed that the VTW peptide specifically increased the uptake of nitric oxide by GBM cells.³⁰ In this study, we showed that the nanoparticles made

of PEI and the VTW peptide significantly and specifically improved the transfection efficiency of GBM cells.

Many techniques, including purification, preparation, and chemical modification, have been used to improve the properties of nanoparticles.^{16,31} A heterobifunctional crosslinker, SPDP, has an amine-reactive radical called the *N*-hydroxysuccinimide ester and a sulfhydryl-reactive radical called 2-pyridyldithio.^{32–34} This crosslinker guaranteed that PEI-PDT and VTW were conjugated accurately. DMF and GnHCl can act as a nonpolar cosolvent and increase the solubility of PEI-PDT conjugated with the VTW peptide.^{35–37} It is also important to note that the peptide solution should

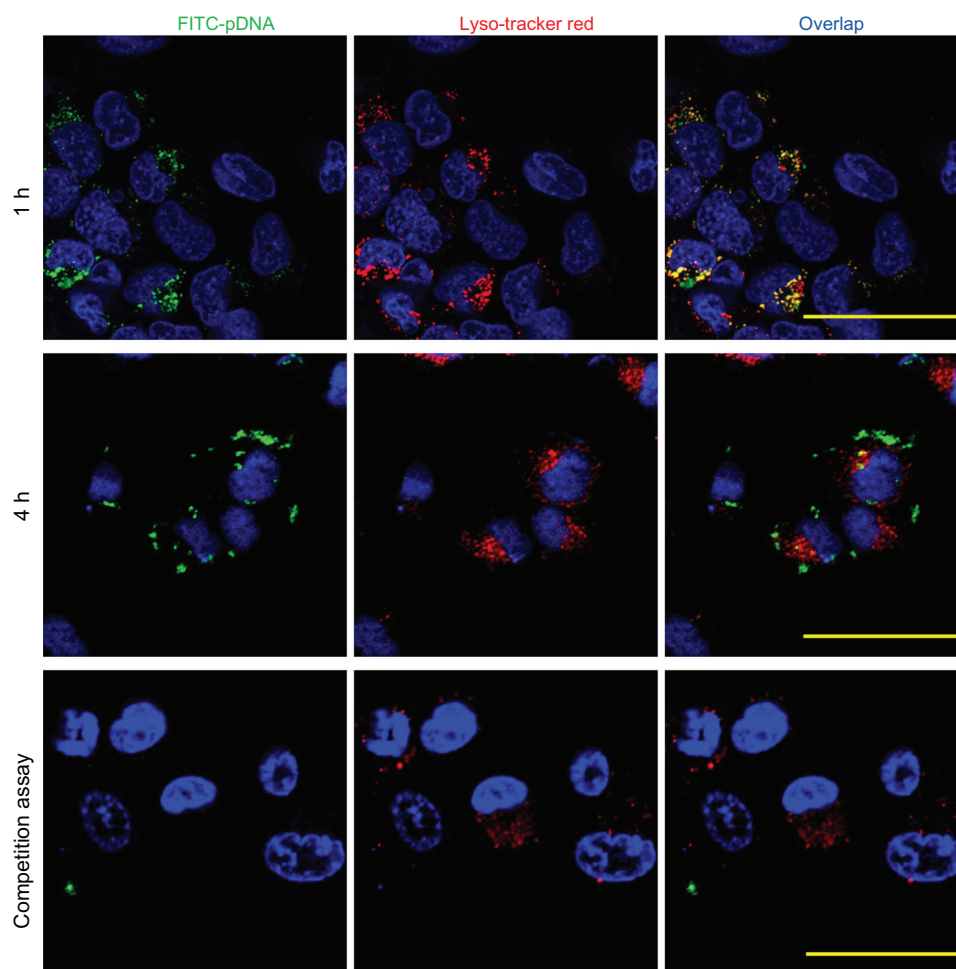


Figure 5 Internalization of nanoparticles.

Notes: U-87 cell was incubated with the PV₂₀ nanoparticles containing the FITC-labeled pDNA. Lysosome was marked with Lyso-Tracker Red (Beyotime, Haimen, People's Republic of China) to investigate the location of nanoparticles in cytoplasm. The nucleus was stained with Hoechst 33342 (Beyotime, Haimen, People's Republic of China). Internalization assays showed that PV₂₀ nanoparticles penetrated into U-87 cells, were mostly co-localized with lysosomes at 1 hour after the beginning of transfection, and escaped successfully from lysosomes at 4 hours. Competition assay showed there was very little internalization of PV₂₀ nanoparticles in U-87 cells due to the block effect by free VTW peptide. Scale bar: 50 μ m.

Abbreviations: FITC, fluorescein isothiocyanate; pDNA, plasmid DNA; PV, VTW-modified PEI copolymers; VTW, VTWTPQAWFQWV.

be slowly added to the PEI-PDT solution with constant vortexing to prevent local, temporary, high concentrations of the peptide. The peptide seemed to form a layer clinging to the surface of the PV nanoparticles, especially PV₂₀ and PV₄₀ (Figure 1 TEM images), which was thought to be the peptide linked with PEI. Due to this peptide-linked copolymer, the surface charge of the nanoparticles was reduced and the diameter was increased.

Successful transfection of polymer-based DNA nanoparticles depends on many factors, including cell internalization, escape from endosomes, DNA release from the nanoparticles, DNA entry into nucleus, and protein expression. Cell internalization is one of the most important parts of transfection, and it can be influenced by cell type, properties of the cationic polymers, size and charge density of the nanoparticles, and the endocytosis mechanism.^{7,11,15,16,22,36}

Receptor-mediated endocytosis can enhance the cellular uptake of nanoparticles.^{11,16,22,31} In our experiments, the U-87 cells could not be transfected effectively with unmodified PEI nanoparticles at an N/P ratio of 10 (Figures 3 and 4). In general, the VTW peptide facilitated the transfection of PEI nanoparticles into U-87 cells via receptor-mediated uptake, while it reduced the cytotoxicity (Figure 4) due to less residual positive charge on the surface of the nanoparticles. One study found that the VTW peptide may bind to gp130 receptor in the glioma cells.²² Competition assays confirmed that the uptake of PEI nanoparticles is specifically mediated by VTW (Figure 5). When the molar ratio of VTW/PEI was 10, the active targeting effect may not have been enough to increase the uptake of nanoparticles significantly, although the surface charge of the nanoparticles was still positive. When the ratio was 20, the VTW peptide was sufficient

to promote the internalization of the nanoparticles but not enough to decrease the escape of the PEI nanoparticles from the lysosomes (Figure 5). However, a much higher ratio of VTW/PEI (eg, 40) might lead to instability of the nanoparticles and less DNA protection from PEI.

The transfection efficiency of PV₂₀ nanoparticles for U-87, T98G and U251 cells was still good after adding FBS into incubation medium, and even better than Lipofectamine for U-87 cells. The transfection efficiency of the PEI nanoparticles was decreased when serum was present in the medium (Figure 4). This phenomenon showed that the PV₂₀ nanoparticles can prevent the influence of serum, possibly due to the uncharged amino residues on the VTW peptide. Meanwhile, these nanoparticles also greatly increased the transfection efficiency for T98G and U251 cells, although not as much as for the U-87 cells (Figures 3 and 4). In the control, the VTW-based nanoparticles did not improve the transfection efficiency for A549, PC12 and the neural cells (Figures 3 and 4).

From another point of view, one previous report showed that there was an increase of DNA release from the acetylated PEI in transfection for cells, due to the acetylation of the amines in PEI.³⁸ As such, we hypothesized that the release of pDNA from the PV₂₀ nanoparticles may be more efficient, due to the replacement of the amines in PEI by peptides. This may be another reason to increase transfection efficiency. In addition, another property of VTW may promote transfection efficiency. VTW is an amphiphilic peptide, which contains eight uncharged, nonpolar amino acids and four uncharged, polar amino acids,²² hence the partially hydrophobic VTW peptide may make a closer link between the nanoparticles and the lipids of the U-87 cells after the nanoparticles are bound by the receptor on the cell membrane that specifically interacts with VTW. The PV₂₀ nanoparticles presented approximately 14-fold higher transfection expression levels in U-87 cells than unmodified PEI nanoparticles, even as high as the effect of Lipofectamine ($P > 0.05$).

In short, the VTW peptide increased the transfection efficiency of nanoparticles and at the same time decreased the cytotoxicity, while the PEI component in copolymers increased the escape from lysosomes by its powerful sponge effect. Furthermore, although PEI is nondegradable in cells, considering the VTW has already targeted the PEI nanoparticles to the glioma cells specifically, leaving the neural cells unaffected, there is no need to be concerned about this. Furthermore, PV nanoparticles have an advantage over viral vectors, due to lack of dangers like the immunogenicity and potential oncogenicity of viral

vectors. Our results show that the GBM cell-targeted PV₂₀ nanoparticles had no transfection activity and almost no cytotoxicity towards the neural cells of rat cerebral cortex. This is good news for the potential use in vivo in future, but whether the nanoparticles are biocompatible when used in vivo is still unknown, and the transfection efficiency is still lower than that of viral vectors. Further studies will be aimed at making our nanoparticles more effective and more compatible in vivo.

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Disclosure

The authors report no conflicts of interest in this work.

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