

A Hybrid Nanovector of Suicide Gene Engineered Lentivirus Coated with Bio reducible Polyaminoglycosides for Enhancing Therapeutic Efficacy against Glioma

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An emerging hybrid nanovector integrating the merits of both viral and nonviral vectors has attracted much attention as the next generation of promising gene vectors to overcome the primary challenge of cancer gene therapy. Due to its inherent advantages, lentivirus (Lv) has been increasingly applied and investigated in medical fields. Herein, a new hybrid nanovector (SS-HPT/Lv) composed of the Lv core and reduction-responsive hyperbranched polyaminoglycoside (SS-HPT) shell is designed via electrostatic interaction. In comparison with polybrene (commercial enhanced transfection reagent), SS-HPT endows the hybrid nanovector with better biosafety. Furthermore, both the appropriate nanoparticle sizes and positive surface potentials contribute to the endocytosis and rescue of SS-HPT/Lv from endocytic vesicles. Regarding therapeutic application, lentiviral vector acquires permanent transgene expression with the capability of integrating to the host chromosome, and SS-HPT/Lv exhibits an improved transduction efficacy. The cytosine deaminase/5-fluorocytosine suicide gene therapy system mediated by SS-HPT/Lv performs an enhanced antitumor efficiency and extends the survival time of glioma-bearing rats. Such a hybrid nanovector strategy would open a new avenue to the development of gene vectors for treating malignant cancers.

in comprehensive treatments, the life expectancy of persons with glioblastoma remains limited and fails to exceed 14–20 months.^[2] Such poor prognosis might be conquered with the improvement of gene therapy. Gene therapy constitutes a promising approach for cancer treatments, which recently has acquired preclinical success. However, the development of appropriate safe and efficient gene vectors is not a straightforward proposition.^[3] Generally, gene vectors mainly include viral and nonviral ones.^[4] Viral vectors possess natively superior transduction efficacy while their inevitable disadvantages impose restrictions on clinical application, including high cost, immunogenicity, and carcinogenesis risk.^[5] In contrast, nonviral vectors could avoid above-mentioned drawbacks.^[6] However, the biggest challenge of nonviral vectors lies in their poor transfection efficiencies.^[7] Thus, a hybrid nanovector strategy has been proposed as one of the burgeoning paradigms, where viral

vectors are encapsulated into hybrid nanoparticles with different nonviral systems to integrate the complementary virtues of both vectors.^[8]

In past few years, some efforts had been made to explore the generation of hybrid nanovectors. Polymer coatings on adenovirus or retrovirus not only enhanced transduction efficiency^[9,10] but also shielded viral vectors to evade immune systems.^[9] Among viral vectors, lentivirus (Lv) has been increasingly applied and investigated due to its inherent advantages such as infecting both dividing and quiescent cells and integrating into host chromosome.^[11] Lv was applied in T-cell engineering ex vivo in CAR-T (Chimeric Antigen Receptor T) cell therapy and the production of autologous corrected CD34⁺ cells ex vivo in patients with β -thalassemia.^[5b,12] Importantly, the transgenes mediated by Lv could be expressed efficiently in animal brain models without detectable pathological consequences, implying a great clinical value in central nervous systems.^[13]

Cytosine deaminase/5-fluorocytosine (CD/5-FC) suicide gene system, as a highly promising approach against cancer, had been investigated extensively,^[14] which could catalyze the conversion of nontoxic 5-FC to highly toxic metabolite 5-fluorouracil (5-FU).^[15] It is quite useful to develop Lv-based hybrid nanovectors that can efficiently deliver CD suicide genes against glioblastoma. Aminoglycosides have already

1. Introduction

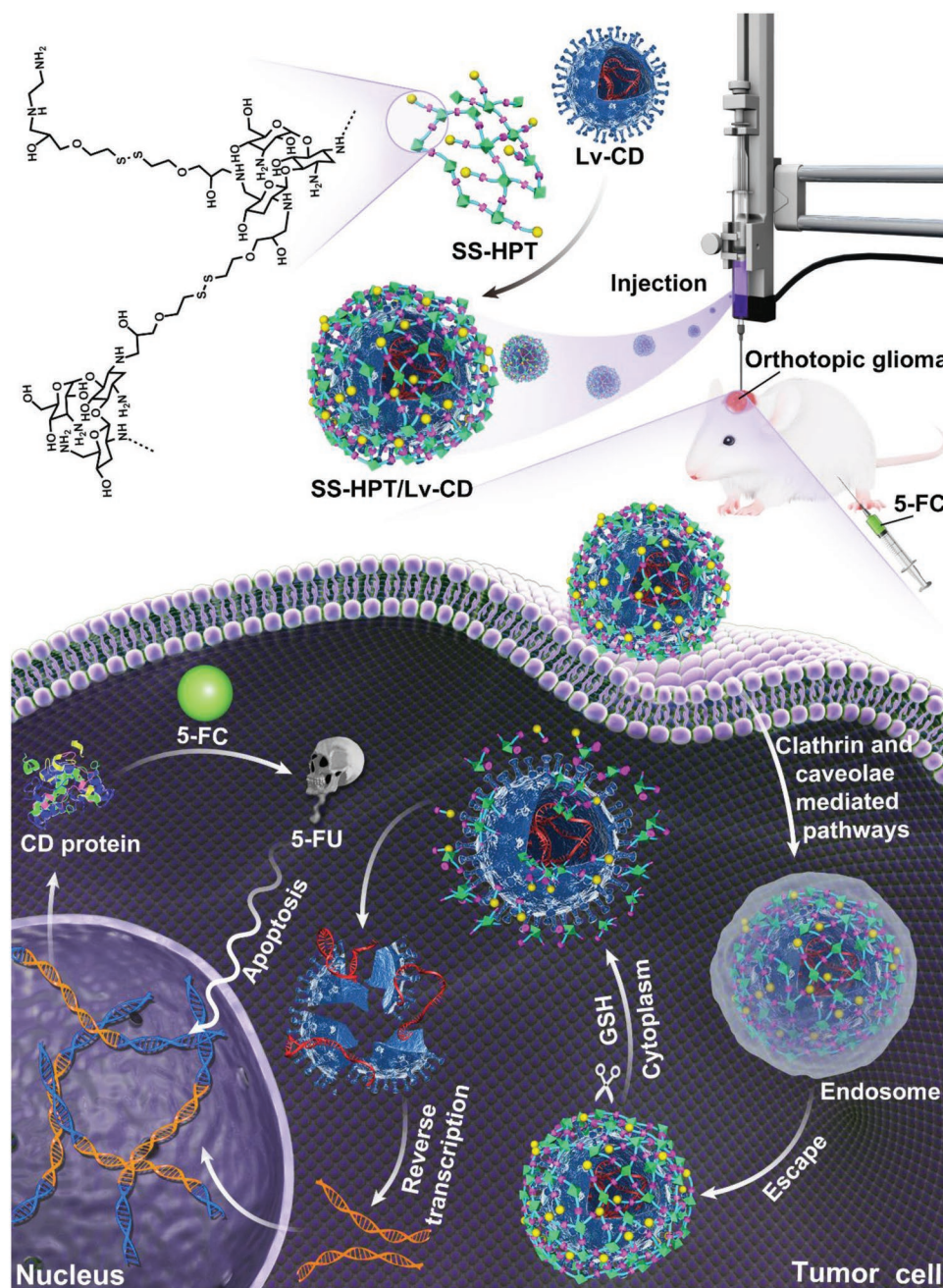
In all cancers, glioblastoma represents the common malignant one with an aggressive growth pattern.^[1] Despite the advances

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Scheme 1. Schematic illustration of the construction of SS-HPT/Lv-CD hybrid nanovector and the resultant therapeutic processes of gene therapy with 5-FC against glioma.

been explored for designing nonviral vectors.^[16,17] Currently, bioreducible hyperbranched polyaminoglycosides (SS-HPT) involving tobramycin were proposed by us,^[17] which exhibited good transfection performance. In this work, a novel hybrid nanovector (SS-HPT/Lv-CD) is readily constructed by coating CD suicide gene engineered Lv (Lv-CD) with SS-HPT (**Scheme 1**) for enhancing therapeutic efficacy against glioma. The reduction-responsive properties of SS-HPT benefit the biodegradability of SS-HPT/Lv-CD. The feasibility against malignant glioma using SS-HPT/Lv-CD nanohybrids was examined in detail via a series of *in vitro* and *in vivo* experiments.

2. Results and Discussion

2.1. Physiochemical Characteristics of SS-HPT/Lv Hybrid Nanovectors

To construct one optimal hybrid nanovector, a series of representative SS-HPT/Lv nanovectors were firstly fabricated using negatively charged Lv and positively charged SS-HPT (with number-average molecular weight of $8.97 \times 10^3 \text{ g mol}^{-1}$) via electrostatic interaction. A range of molar ratios for SS-HPT to Lv from 3.3×10^7 to 8.3×10^8 molecules per Lv

particle were investigated using different concentrations of SS-HPT solutions of 2 to 50 $\mu\text{g mL}^{-1}$ (Table S1, Supporting Information). The corresponding SS-HPT/Lv nanovectors were denoted as SS-HPT/Lv-2 (3.3×10^7 molecules per Lv), SS-HPT/Lv-5 (8.3×10^7 molecules per Lv), SS-HPT/Lv-10 (1.7×10^8 molecules per Lv), SS-HPT/Lv-20 (3.3×10^8 molecules per Lv), and SS-HPT/Lv-50 (8.3×10^8 molecules per Lv), respectively.

The hydrated particle sizes and zeta potentials of SS-HPT/Lv nanovectors were measured using dynamic light scattering (DLS) (Figure 1a,b). The average size of SS-HPT/Lv increased to $423 (\pm 23.8)$ nm from $209 (\pm 20.3)$ nm of naked Lv initially, then decreased gradually with increasing the amount of SS-HPT. A stable status of about 300 nm was reached for SS-HPT/Lv-20. The surface zeta potential of SS-HPT/Lv increased gradually to a peak of $9.8 (\pm 0.6)$ mV at 3.3×10^8 molecules per Lv of SS-HPT/Lv-20 compared to $-15.7 (\pm 1.6)$ mV of naked Lv.

These data suggested that Lv particles could be compacted better into one appropriate nanoparticle with positive surface potential, which would provide the good affinity between Lv and cell surfaces.^[9a,d] Meanwhile, the atomic force microscope (AFM, Figure 1c) and transmission electron microscope (TEM, Figure 1d) images also showed that the particle size of SS-HPT/Lv-20 was larger than that of naked Lv. The original sphere morphology of Lv remained.

Biodegradability represents another crucial parameter of gene vectors. SS-HPT with disulfide bonds in its backbone could be degraded by reducible molecules such as glutathione (GSH).^[18] Polymers with disulfide bonds were designed as gene vectors, aiming at facilitating the release of target gene from polyplexes and reducing the cytotoxicity of vectors.^[9d,17,18] The content of GSH in tumor cells is several times higher than that in normal cells, which is one of many characteristic aspects

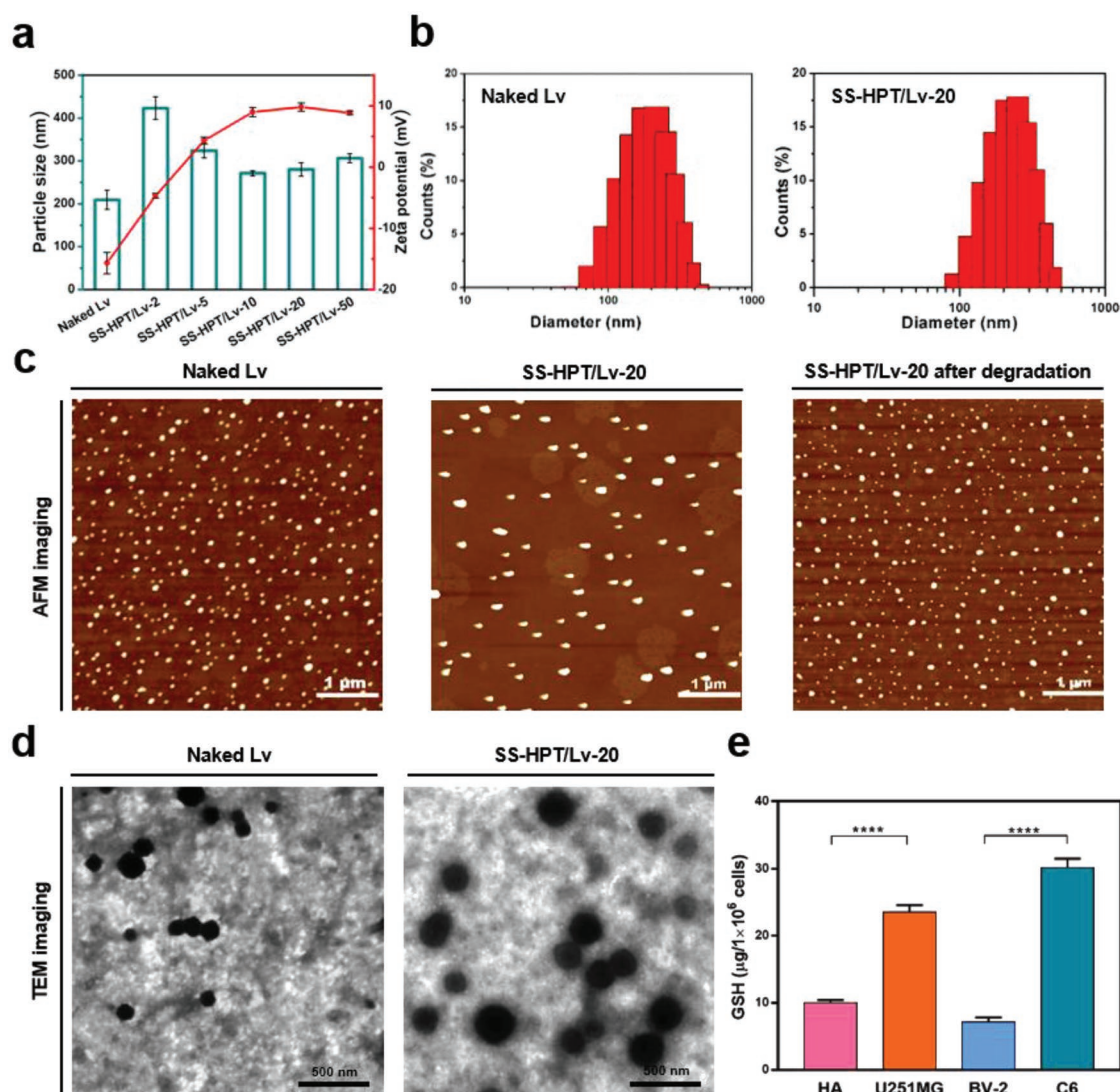


Figure 1. a) Particle size and zeta potential of naked Lv and SS-HPT/Lv hybrid nanovectors at 3.3×10^7 , 8.3×10^7 , 1.7×10^8 , 3.3×10^8 , and 8.3×10^8 molecules per Lv for SS-HPT/Lv-2, SS-HPT/Lv-5, SS-HPT/Lv-10, SS-HPT/Lv-20, and SS-HPT/Lv-50, respectively ($n = 5$). b) Size distribution of naked Lv and SS-HPT/Lv-20. c) Representative AFM images of naked Lv and SS-HPT/Lv-20 before or after degradation. d) Representative TEM images of naked Lv and SS-HPT/Lv-20. e) GSH contents in U251MG and C6 glioma cells in comparison with normal HA and BV-2 brain cell lines ($n = 3$, **** $p < 0.0001$).

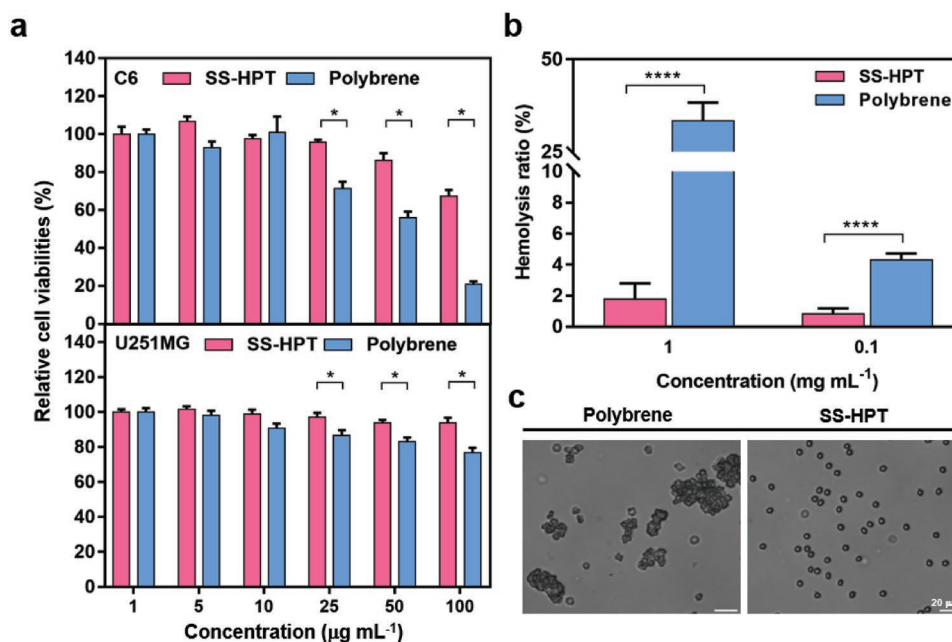


Figure 2. a) Cell viabilities of SS-HPT and polybrene in C6 and U251MG cell lines ($n = 5$, $*p < 0.05$). b) Hemolysis ratios of RBCs treated with SS-HPT or polybrene at the concentrations of 1 and 0.1 mg mL⁻¹ ($n = 3$, $****p < 0.0001$). c) Representative images of depositional RBCs treated with polybrene or SS-HPT at 1 mg mL⁻¹.

in tumor cells.^[19] As shown in Figure 1e, the contents of GSH in the corresponding C6 and U251MG glioma cells were approximately 4.2- and 2.3-folds compared to normal brain cells, which would endow SS-HPT/Lv with better performance in glioma cells.^[19c] After degradation (Figure 1c), the homogeneous SS-HPT/Lv nanovector returned to the similar morphology to naked Lv. The above results indicate that SS-HPT/Lv would be degraded readily in glioma cells with higher GSH contents.

2.2. Biophysical Properties of SS-HPT/Lv Nanovectors

To achieve the optimal coating ratio of SS-HPT, it is also necessary to clarify the cytotoxicity (Figure 2a and Figure S1a, Supporting Information) of SS-HPT. Similar to our earlier work,^[17] SS-HPT exhibited a slight dose-dependent cytotoxicity in glioma cell lines, as well as in normal brain cell lines. More importantly, SS-HPT showed evidently lower cytotoxicity than the commercially available enhanced transfection reagent, polybrene in all cell lines. It is noteworthy that cell viability of SS-HPT exceeded 70% even at the higher concentration (100 µg mL⁻¹) for all cell lines, which was consistent with our earlier results.^[17]

For gene therapy, blood compatibility of vectors also requires major concerns.^[20] Biomaterials were regarded to be hemolytic according to the hemolysis ratio of $>5\%$.^[20b] The quantitative hemolysis ratios of SS-HPT were $0.81 (\pm 0.4)\%$ and $1.78 (\pm 1.0)\%$ at the concentrations of 0.1 and 1 mg mL⁻¹, respectively, considerably lower than those of polybrene (Figure 2b). Furthermore, the supernatants of red blood cell (RBC) suspensions in the SS-HPT group were almost transparent (Figure S1b, Supporting Information), indicating that SS-HPT was eligible for the application standard of biomaterial ($<5\%$).^[20c] The extent of erythrocytes aggregation was considered as another criterion

for blood compatibility (Figures 2c and Figure S1c, Supporting Information). In comparison to polybrene, no obvious erythrocytes coagulation was detected in the SS-HPT group. Polybrene is an effective neutralized agent of heparin during open-heart surgery.^[21] However, its antiheparin property reduced the biocompatibility as an enhanced transduction reagent. These above results demonstrate that SS-HPT possessed a preponderance of blood biocompatibility over polybrene.

To determine the optimal SS-HPT ratio for Lv coating, the transduction efficiency of reporter gene ZsGreen was utilized as an indicator. C6 and U251MG glioma cell lines with different sensitivities for Lv were selected. They expressed different levels of the receptors (such as phosphatidylserine, low density lipoprotein receptor or leucine-rich repeat-containing G protein-coupled receptor 4) of vesicular stomatitis virus (VSVG), a trimeric protein used to pseudotype the envelope in Lv vector.^[22] According to the representative fluorescence images of ZsGreen expressions, SS-HPT/Lv displayed a gradually enhanced tendency in the transduction performance in both cell lines (Figure 3a). Furthermore, the quantitative fluorescence-activated cell sorting (FACS) analysis shows that the highest ZsGreen intensity was observed for SS-HPT/Lv-20 (with the ratio of 3.3×10^8 molecules per Lv) in both glioma cell lines (Figure 3b). The optimal transduction efficiencies were increased by 2.56- and 1.73-folds compared to naked Lv, respectively. The above results indicated the optimal Lv coating ratio was at 3.3×10^8 molecules per Lv. Thus, SS-HPT/Lv20 was selected in the subsequent experiments.

The effect of serum on transduction efficiency was also investigated for better in vivo applications. As shown in Figure S2 in the Supporting Information, the transduction efficiency of naked Lv was obviously reduced in the presence of serum. On the contrary, no distinct difference was detected

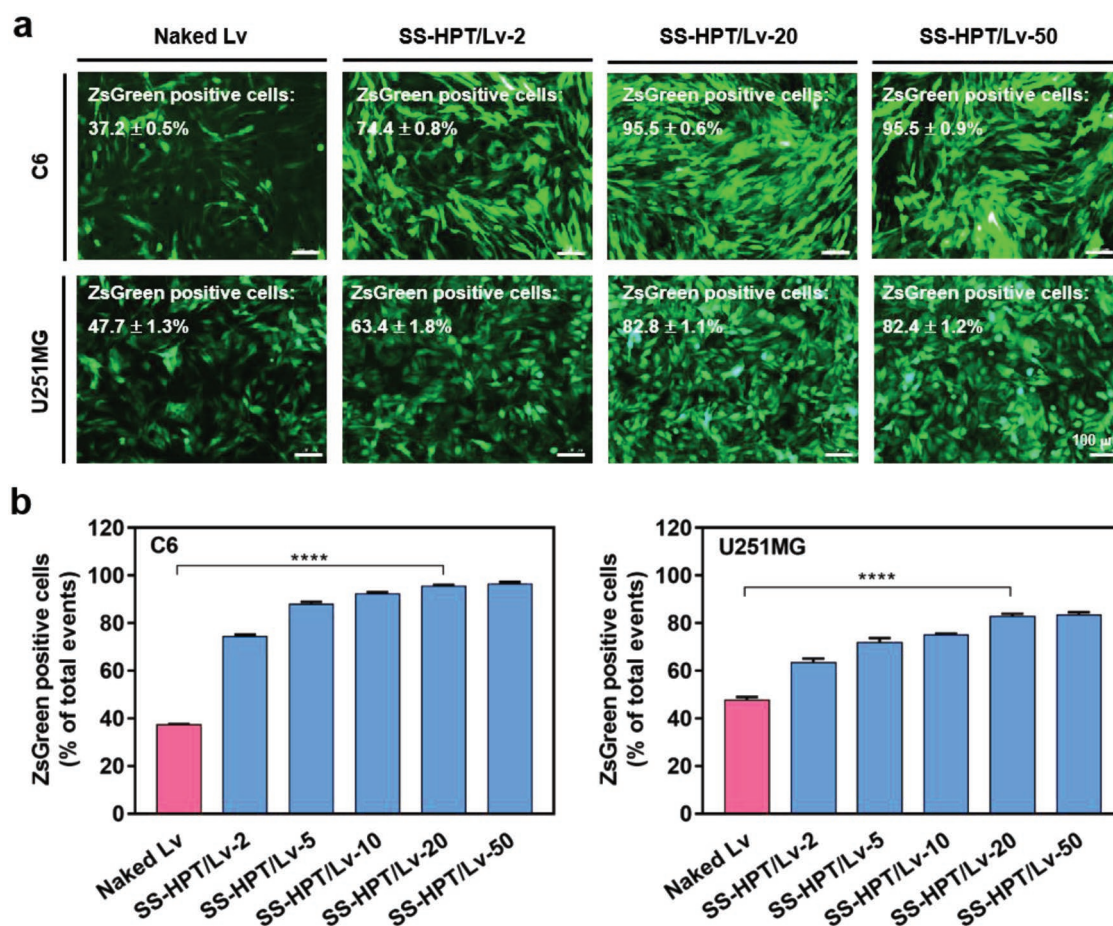


Figure 3. a) Representative fluorescence images and b) quantitative FACS analysis of ZsGreen expression ($n = 3$, **** $p < 0.0001$) mediated by naked Lv or SS-HPT/Lv nanovectors at different ratios of SS-HPT to Lv in C6 and U251MG cell lines.

in the SS-HPT/Lv-20 group. We further tested the stability of SS-HPT/Lv-20 in medium with serum and found that the particle size of the hybrid nanovector remained quite stable during the incubation (Figure S3, Supporting Information). SS-HPT possesses plentiful hydroxyl groups. Our earlier work demonstrates that rich nonionic hydrophilic hydroxyl species not only can shield the deleterious surface charges of complexes but also reduce protein adsorption on complexes for enhancing gene expression.^[3c,6b,17] The above data indicated that SS-HPT/Lv exhibited the good serum-tolerant ability.

2.3. Cellular Uptake Mechanism of SS-HPT/Lv

As Lv infectivity is dependent on the VSVG receptors expressed on cell membranes,^[22b] the responsible cellular uptake mechanism of SS-HPT/Lv was explored in comparison with naked Lv after the pretreatment with inhibitors of different endocytic pathways in C6 cell line.^[23] Methyl- β -cyclodextrin (M β CD) was used to stymie caveolae-mediated endocytosis by selectively extracting cholesterol from cell membranes,^[24] while cytochalasin B (CytoB) and ammonium chloride (NH₄Cl) were considered as the corresponding inhibitors of clathrin-mediated endocytosis pathway and phagosome-lysosome

fusion.^[25] As shown in Figure 4a,b, both M β CD and CytoB significantly reduced the transduction efficiencies mediated by SS-HPT/Lv-20, while CytoB did not cause any influence on naked Lv-mediated transduction. SS-HPT/Lv-20 appeared to induce both caveolae- and clathrin-mediated endocytosis, while naked Lv was primarily internalized into cells through caveolae-mediated endocytosis. The transduction efficiency was inhibited by NH₄Cl in both cases of naked Lv and SS-HPT/Lv-20, indicative of the common involvement of phagosome-lysosome fusion. These above results demonstrate that SS-HPT/Lv-20 entered cells via a broader route than naked Lv, followed by fusing with lysosome to unleash Lv particles efficiently.

The details of intracellular trafficking were tracked at early time points (10, 30, and 120 min) using TEM (Figure 4c). In comparison with naked Lv, more SS-HPT/Lv-20 nanoparticles were observed around the cell membrane at 10 min and internalized into the cytoplasm at 30 min. After 120 min post-transduction, a single endocytic vesicle containing one nanoparticle was observed in the cytoplasm of the naked Lv group. However, plenty of single endocytic vesicles containing multiple nanoparticles were localized near the nucleus in the SS-HPT/Lv-20 group. The better transduction performance of SS-HPT/Lv-20 was probably due to both the broader cellular uptake route and more efficient intracellular trafficking.

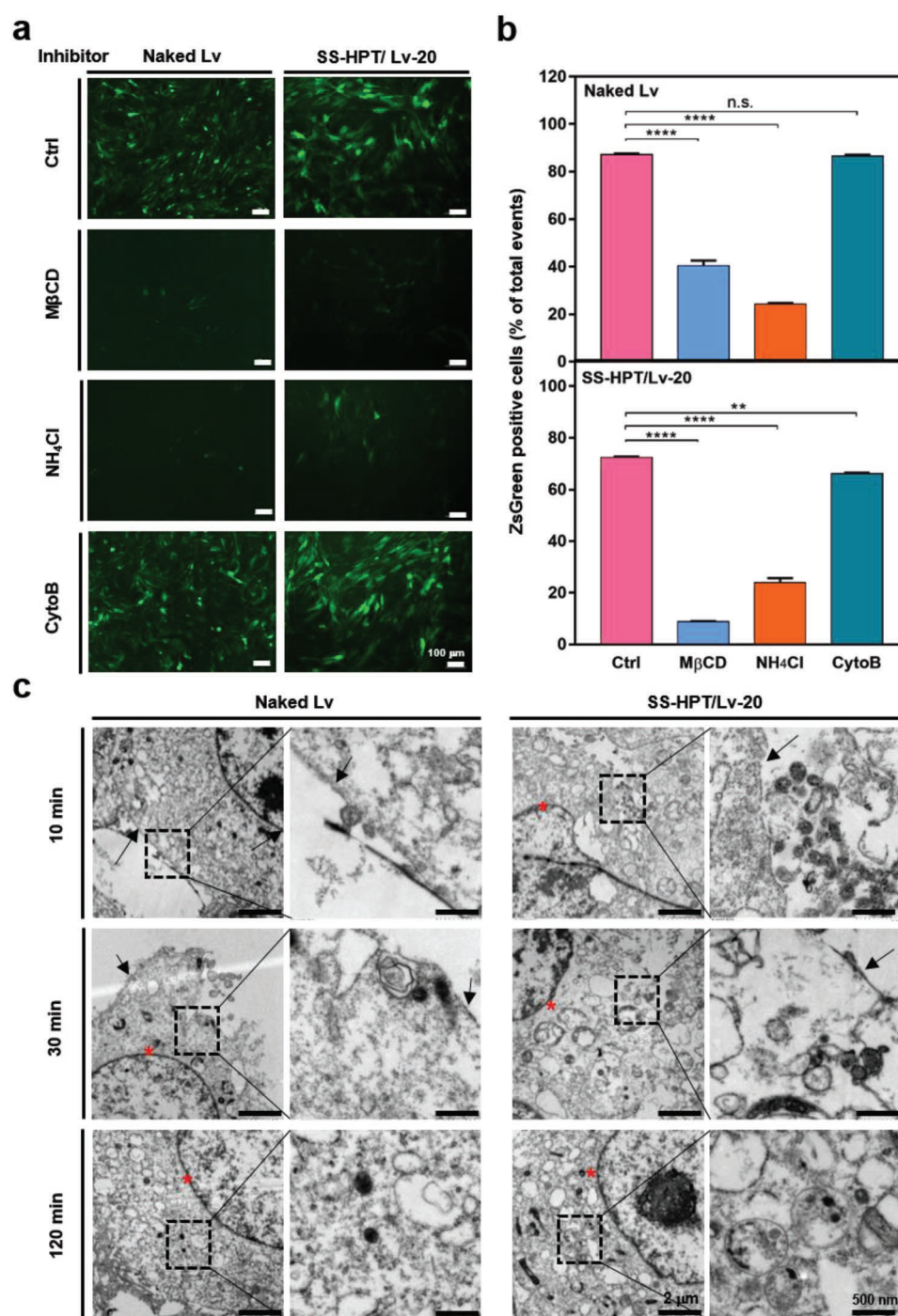


Figure 4. a) Representative fluorescence images and b) quantitative FACS analysis of the ZsGreen expression in C6 cells mediated by naked Lv or SS-HPT/Lv-20 in the presence of various endocytic inhibitors ($n = 3$, $**p < 0.01$, $****p < 0.0001$). c) Representative TEM images of intracellular trafficking of naked Lv or SS-HPT/Lv-20 at indicated time points (black arrow: cell membrane, red star: nuclear envelop).

2.4. Enhanced Transduction Efficiency of SS-HPT/Lv-CD

As a proof of concept, Lv-CD (carrying reporter gene ZsGreen and CD suicide gene simultaneously) was employed to construct the SS-HPT/Lv-CD hybrid nanovector at the optimal coating ratio of SS-HPT, where polybrene coated Lv-CD was taken as the positive control. As shown in Figure 5a, SS-HPT/Lv-CD mediated

much stronger ZsGreen intensities than naked Lv-CD in both glioma cells. In general, it is more difficult for gene vector to efficiently deliver larger gene. The transduction efficiencies (demonstrated by ZsGreen positive cells, e.g., 3.7% for naked Lv-CD and 38.0% for SS-HPT/Lv-CD in C6 cells) of Lv-CD are significantly lower than those (e.g., 37.2% for naked Lv and 95.5% for SS-HPT/Lv in C6 cells) of Lv (Figure 3). The

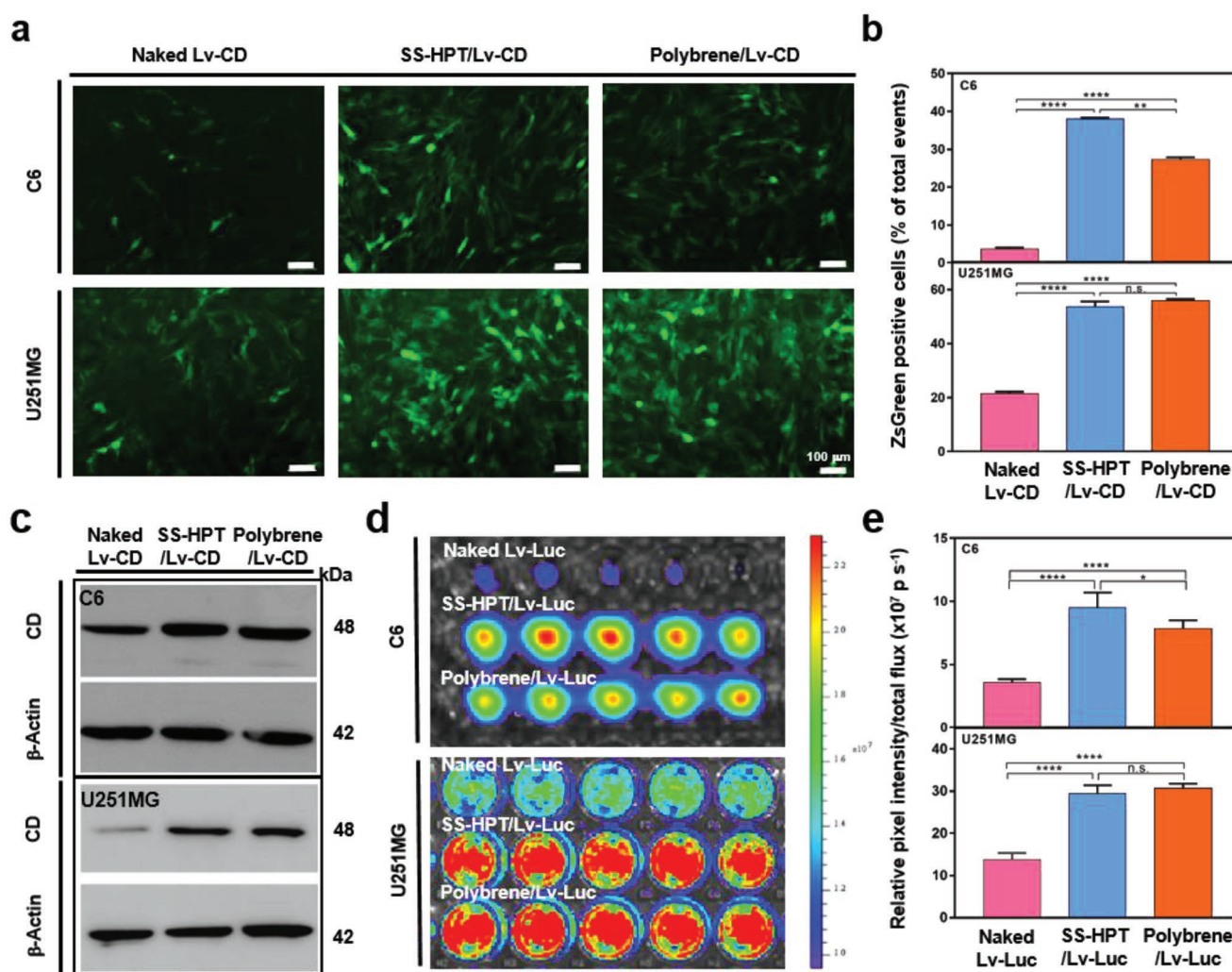


Figure 5. a) Representative fluorescence images and b) quantitative FACS analysis of ZsGreen expression mediated by naked Lv-CD, SS-HPT/Lv-CD and polybrene/Lv-CD in C6 and U251MG cell lines ($n = 3$, **** $p < 0.0001$). c) Relative CD expression determined by western blot analysis where β -actin was as the internal reference. d) Representative bioluminescence images and e) quantitative luciferase pixel intensity mediated by Lv-Luc or SS-HPT/Lv-Luc, and polybrene/Lv-Luc ($n = 5$, **** $p < 0.0001$).

transduction efficiency of SS-HPT/Lv-CD increased by 10.2- and 2.5-folds relative to naked Lv-CD in C6 and U251MG cell lines, respectively (Figure 5b). The transduction efficiency of SS-HPT/Lv-CD was striking in C6 glioma cells, probably attributed to the higher up-regulated GSH level in C6 cell line (Figure 1e).

To directly confirm the enhanced transduction performance, the CD protein expression was analyzed using western blotting assay (Figure 5c and Figure S4, Supporting Information). As expected, the SS-HPT/Lv-CD group obviously increased CD protein expression compared with naked Lv-CD. Furthermore, as shown in Figure S5 in the Supporting Information, CD protein was initially expressed at 12 h post-transduction and the expression peak occurred at 48 h post-transduction in all three groups, confirming that SS-HPT did not interfere with the CD protein expression pattern. Importantly, SS-HPT/Lv-CD could compensate for the insufficiency of Lv for gene delivery.

Subsequently, to further verify the enhanced transduction efficiency of the hybrid nanovector, luciferase engineered

Lv (Lv-Luc) as another reporter gene was utilized to construct a hybrid nanovector (SS-HPT/Lv-Luc). Similar to the above case of SS-HPT/Lv-CD in C6 cell line, the strongest luciferase signal was observed in the SS-HPT/Lv-Luc group (Figure 5d,e). It was well-known that C6 glioma cell was hard to be infected with Lv because of lower VSVG receptors. The above data confirmed that SS-HPT/Lv can improve gene transduction efficiency, especially for Lv refractory infected cells.

2.5. Improved Chemosensitivity of 5-FC Mediated by SS-HPT/Lv-CD in Vitro

Based on its above good transduction performance, SS-HPT/Lv-CD could enhance glioma cell-killing effect with 5-FC in vitro. As shown in Figure 6a, a remarkable increase in glioma cell death was observed within different 5-FC concentrations. More importantly, in comparison with the naked Lv-CD and

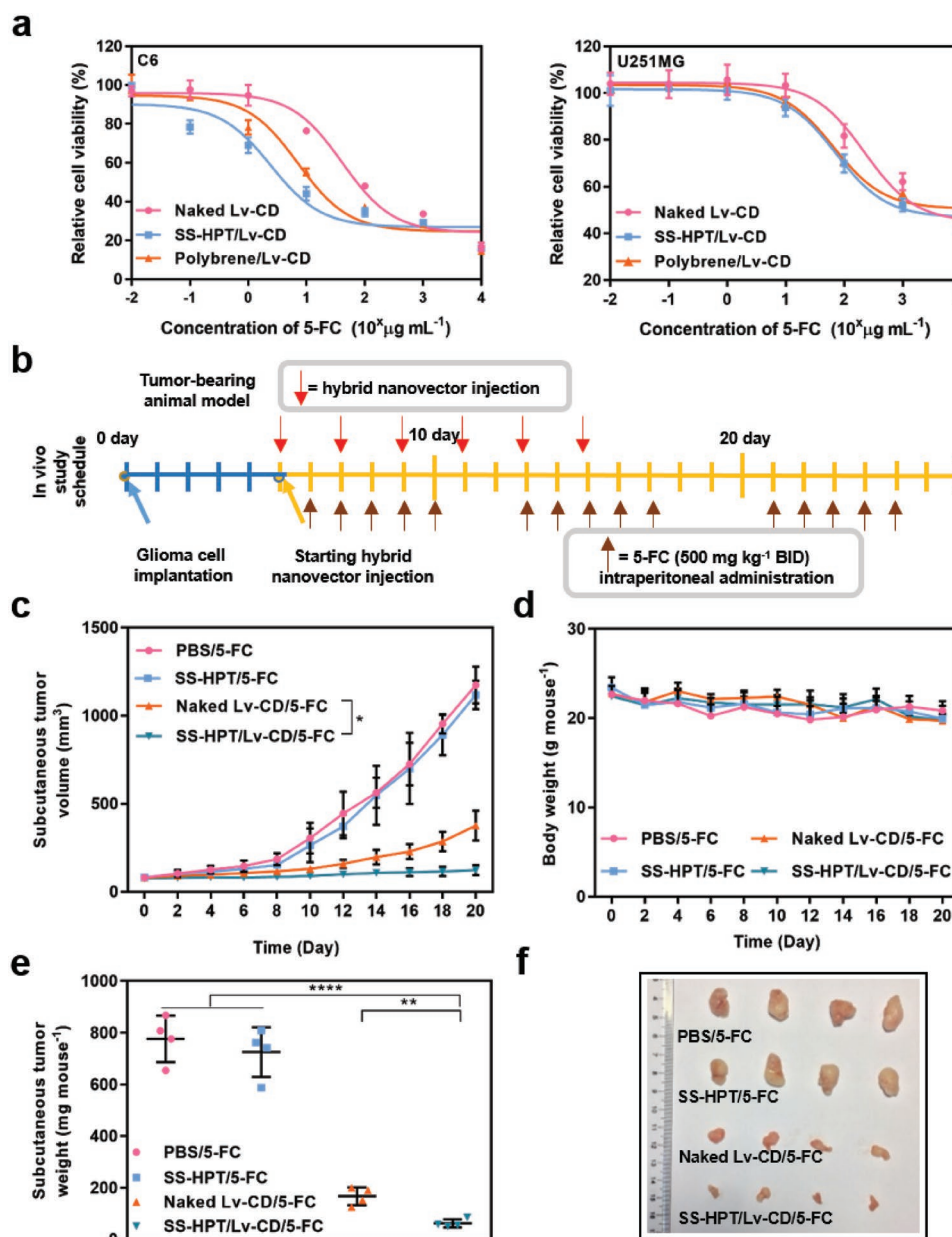


Figure 6. a) Glioma cell killing effect induced by naked Lv-CD, SS-HPT/Lv-CD, and polybrene/Lv-CD with different concentrations of 5-FC in C6 and U251MG cell lines in vitro ($n = 3$). b) Schedule illustration of in vivo study. c) Time-dependent tumor growth curves of mice after treatment with PBS, SS-HPT, naked Lv-CD, and SS-HPT/Lv-CD with 5-FC ($n = 4$, * $p < 0.05$). d) Body weight of mice during the treatment period. e) Weights and f) photographs of excised subcutaneous tumors in different groups ($n = 4$, ** $p < 0.01$, *** $p < 0.0001$).

polybrene/Lv-CD groups, a higher level of glioma cell death was induced in the SS-HPT/Lv-CD group.

By calculating the IC_{50} values of 5-FC in three groups (Table S2, Supporting Information), it was found that the IC_{50} value of 5-FC in the SS-HPT/Lv-CD group was smallest. The conversion of toxic 5-FU from 5-FC catalyzed by CD enzyme is in a time-dependent manner and about 86% conversion needs about 6 days.^[26] Thus, there remained survival glioma cells even at an extreme dosage ($10\,000 \mu\text{g mL}^{-1}$). It should be noted that the CD/5-FC suicide system mediated by SS-HPT/Lv-CD also exhibited a pre-eminent antitumor effect in relatively short time (72 h).

2.6. Enhanced Antitumor Efficacy of SS-HPT/Lv-CD with 5-FC in Vivo

Encouraged by the excellent in vitro antitumor effect mediated by SS-HPT/Lv-CD, we further proceeded to evaluate the potential therapeutic effect against glioma using pre-established xenografts models (Scheme 1). C6 cell lines exhibit an aggressive growth pattern, which could recapitulate the hallmarks of clinical glioma.^[14e,27] After the treatment with naked Lv-CD (or SS-HPT/Lv-CD) and 5-FC by intraperitoneal (i.p.) administration (Figure 6b), the tumor volume curve

showed an decelerated tumor growth in the naked Lv-CD and SS-HPT/Lv-CD groups. Particularly, the SS-HPT/Lv-CD group produced the best antitumor performance (Figure 6c), which was in agreement with the enhanced glioma killing effect in vitro (Figure 6a). The animal body weight loss represents a toxic indicator of gene vectors.^[18] As shown in Figure 6d, all four groups had no obvious negative impact throughout the experiment period, indicating the good in vivo biocompatibility of SS-HPT/Lv-CD. At the therapeutic endpoint, the mice were sacrificed and the excised tumors were weighted and captured (Figure 6e,f), where the tumor weight in the Lv-CD group approached nearly 2.7 times than that in the SS-HPT/Lv-CD group (167.6 ± 34.3 g vs 62.9 ± 15.6 g). The minimal tumor was observed in the SS-HPT/Lv-CD group, further confirming the good antitumor performance of SS-HPT/Lv-CD.

Regarding the underlying mechanism of different treatments at the molecular level (Figure 7), the hematoxylin and eosin (H&E) staining reveals that more shrunken or fragmented cells were observed in the SS-HPT/Lv-CD group than in other groups. More CD protein expression was also observed in the SS-HPT/Lv-CD group, which provided additional evidences for the enhanced transduction efficiency. The decreased expression level of proliferating cell nuclear antigen (PCNA) indicated the lower cell proliferation

ability in the SS-HPT/Lv-CD groups.^[28] Moreover, terminal deoxynucleotidyl transferase dUTP-nick-end-labeling (TUNEL) assay demonstrates that a greater proportion of apoptotic cells were induced by the SS-HPT/Lv-CD group. The accumulating evidences confirm that the apoptosis induced by 5-FC/CD suicide gene therapy involves the mitochondrial pathway and activated caspase cascades.^[29]

Subsequently, in situ therapeutic assessment was performed using the intracranial orthotopic glioma xenograft model (Figure 8a). The rats received in situ intracranial injections of PBS, SS-HPT, naked Lv-CD, or SS-HPT/Lv-CD, followed by the 5-FC administration. As shown in Figure 8b, no statistically significant difference was observed between the SS-HPT and PBS groups. However, the SS-HPT/Lv-CD group exhibited the minimum tumor volume, and the reduced tumor volume was almost by half of the naked Lv-CD group on the 24th day. Furthermore, the median survival periods of the four groups were 23.5, 24, 33.5, and 39.5 days, respectively (Figure 8c). The Log-rank analysis reveals that the survival time was prolonged in the SS-HPT/Lv-CD group ($p = 0.026$). These in situ antitumor results also demonstrate that SS-HPT/Lv-CD could efficiently deliver target gene and produce stronger in vivo antitumor effects. All the above in vivo results confirm that the SS-HPT/Lv-CD nanovector represents a promising prospect for glioma in gene therapy field.

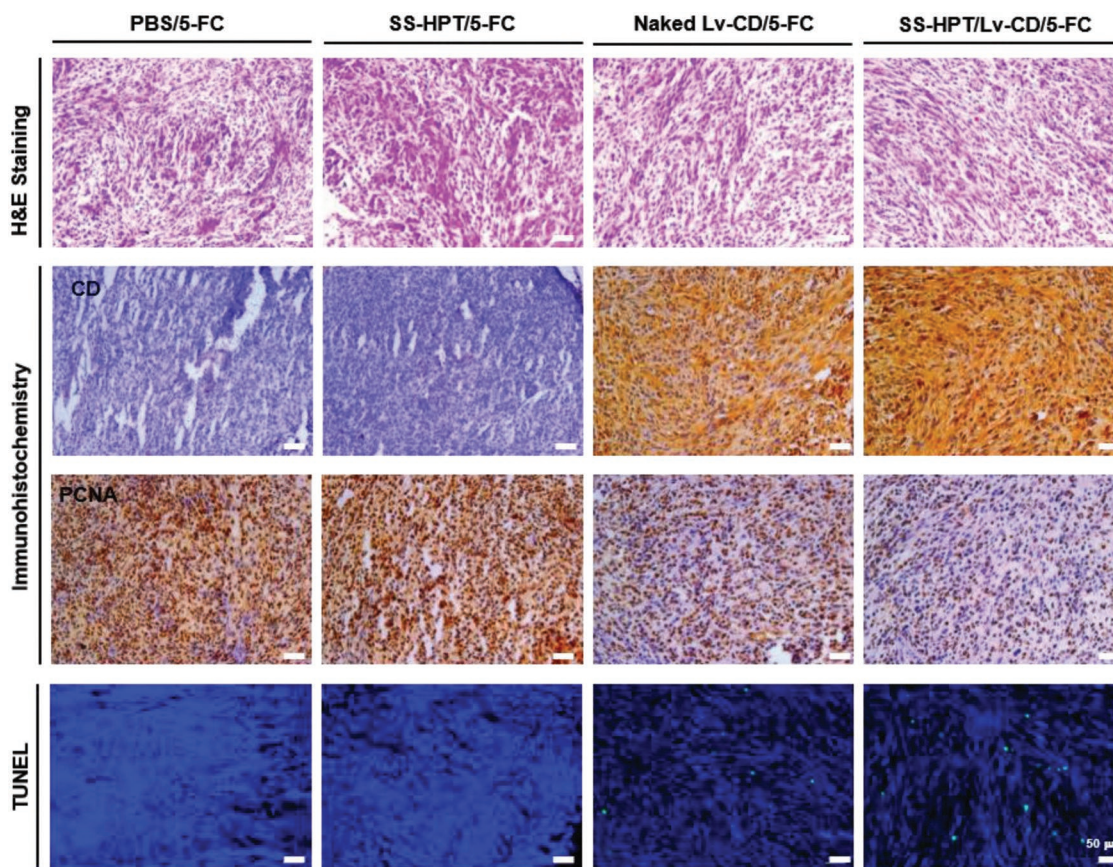


Figure 7. Representative images of dissected tumors stained with H&E, CD, PCNA, and TUNEL after different treatments.

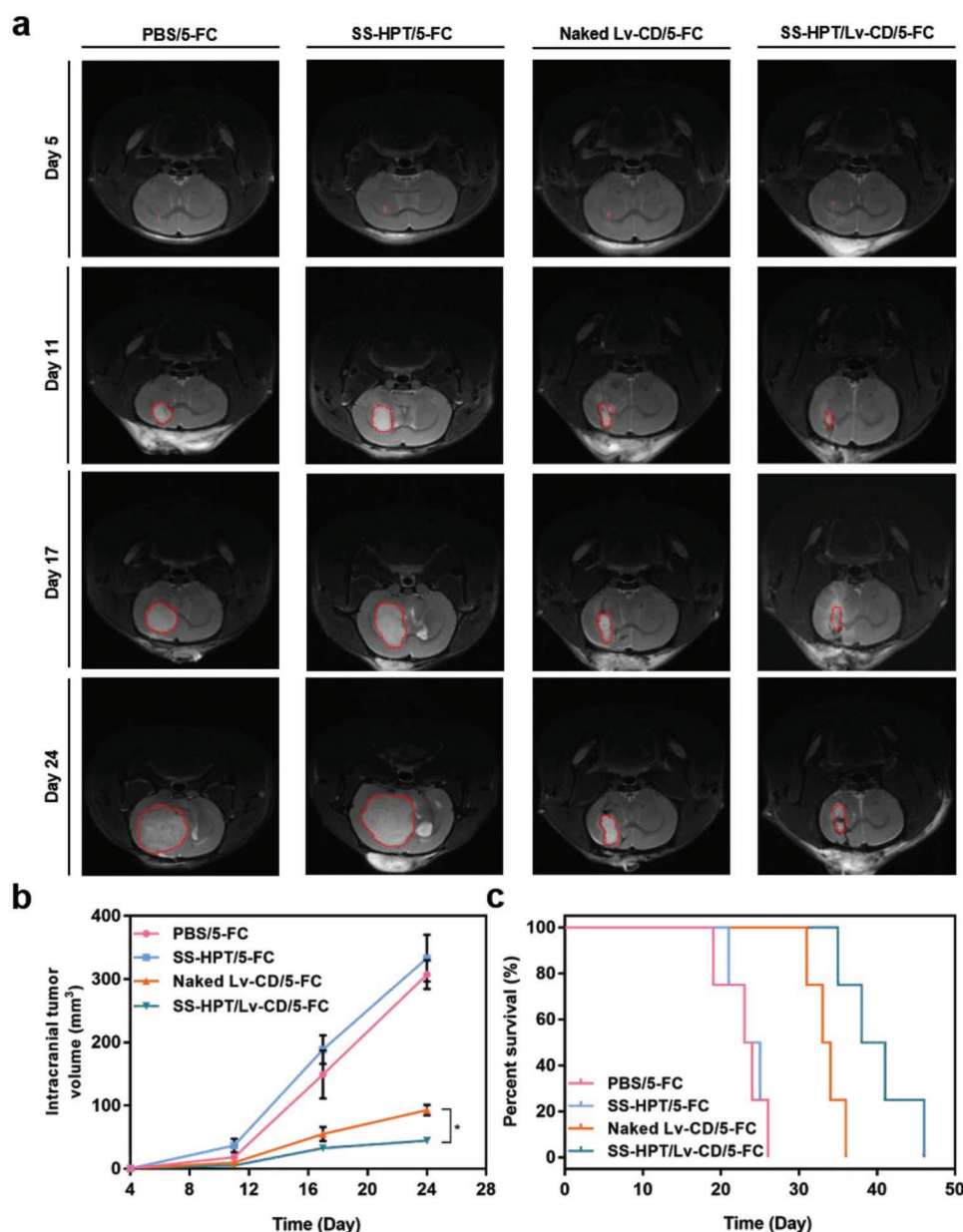


Figure 8. a) Representative T2WI images of intracranial tumors monitored by MRI on Day 5–24 after C6 cell implantation with different treatments. b) Quantitative analysis of tumor volume based on longitudinal T2WI ($n = 4$, $*p < 0.05$) and c) survival curve of glioma-bearing rats ($n = 4$, $p = 0.026$).

3. Conclusions

In summary, a novel SS-HPT/Lv hybrid nanovector was prepared successfully through coating suicide gene engineered Lv with bioreducible SS-HPT. SS-HPT/Lv exhibited good biocompatibility and improved transduction performance as well as effective antitumor effect in vitro and in vivo. Due to both caveolae-dependent and clathrin-mediated endocytosis induced by SS-HPT/Lv-CD, the resulting more CD protein expression led to an enhanced antitumor effect with administration of 5-FC. Furthermore, SS-HPT/Lv-CD obviously improved the chemosensitivity of glioma cells to 5-FC and significantly prolonged the animal survival time of glioma-bearing rats.

This study would provide a feasible avenue toward the development of new hybrid nanovectors with both advantages of viral and nonviral systems against glioma or other refractory solid cancers.

4. Experimental Section

Cell Culture and Lv Generation: C6 rat glioma, U251 human glioblastoma, BV-2 normal mouse microglia, and human embryonic kidney (HEK293T) cell lines were purchased from the Cell Bank of Peking Union Medical College (Beijing, China), which were cultured in DMEM (Invitrogen) media with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Normal human astrocytes cell line

(HA) was purchased from ScienCell Research Laboratories and maintained in astrocyte media (AM) containing astrocyte growth supplement (AGS) with 2% FBS and 1% penicillin/streptomycin (ScienCell, Carlsbad, CA). All cells were cultured in a 37 °C humidified incubator with 5% CO₂. A third-generation packing system was employed to construct all the recombinant Lv vectors (Lv, Lv-CD, and Lv-Luc) as described in earlier work.^[14e] The detailed procedures can be found in Supporting Information.

Preparation and Physicochemical Characterization of SS-HPT/Lv: The ring opening polymerization between hydroxyethyl disulfide diglycidyl ether and tobramycin resulted in the synthesis of hyperbranched SS-HPT (with number-average molecular weight of 8.97×10^3 g mol⁻¹) end-capped with ethylenediamine as described previously.^[17] SS-HPT/Lv hybrid nanovectors were fabricated by adding Lv (4×10^6 TU mL⁻¹) dropwise into the pre-diluted SS-HPT solutions (2, 5, 10, 20, 50 µg mL⁻¹) under the serum-free DMEM condition for 30 min standing at room temperature before use.

The particle size and zeta potentials of SS-HPT/Lv and naked Lv were analyzed in the double distilled water or in the culture medium with or without 10% FBS via a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). In addition, the morphological changes at the optimal ratio before or after NaBH₄ (100 mM) degradation were observed with atomic force microscope (AFM, Bruker, USA) and transmission electron microscope (TEM, H-7650, Hitachi, Japan) by a negative stain technique. The detailed procedure of TEM imaging can be found in Supporting Information.

Biophysical Characterization of SS-HPT/Lv Hybrid Nanovector: The cytotoxicity of SS-HPT was evaluated using a cell counting kit-8 assay (CCK-8, Dojindo Molecular Technologies, Shanghai, China). Transduction efficiency analysis was performed in both C6 and U251MG glioma cell lines by virtue of Lv or Lv-CD, both of which carried a reporter gene, ZsGreen. Commercially available polybrene (Sigma Aldrich, USA) coated Lv-CD was used as the positive control. The cellular uptake mechanism and serum stability were evaluated based on the ZsGreen fluorescence intensity using a fluorescence microscope (Zeiss, Oberkochen, Germany). The ZsGreen positive ratio was quantified by FACS (Beckman Coulter, Germany). The above detailed procedures and other assays (including hemocompatibility, measurement of GSH, TEM imaging of cells, bioluminescence images using Lv-Luc, and western blotting) can be found in Supporting Information.

Antitumor Efficacy of SS-HPT/Lv-CD with 5-FC in Vitro and in Vivo: A cell counting kit-8 assay was utilized to evaluate the cell apoptosis induced by Lv-CD in C6 and U251MG cell lines in vitro. The glioma cells prepared on the previous day were treated with naked Lv-CD, SS-HPT/Lv-CD, or polybrene/Lv-CD. For antitumor efficiency in vivo using subcutaneous and intracranial C6 glioma-bearing animal models, four groups of C6-bearing mice or rats were treated with PBS, pure SS-HPT, naked Lv-CD, or SS-HPT/Lv-CD, respectively, followed by administration i.p. with 5-FC. All the animal experiments were approved by Experimental Animal Ethics Committee of Beijing Neurosurgical Institute and performed under legal protocols. The H&E, histological analysis and TUNEL (Promega, USA) assay of the tumors were performed. The detailed procedures are shown in Supporting Information.

Statistical Analysis: All experiment data were expressed as means ± standard deviation (SD). Student's *t*-test was used between two sets and One-way ANOVA with Bonferroni's correction was used among more than two groups in GraphPad (Version 7). The overall survival time was compared using Log-rank method. *p* < 0.05 was considered to be statistically significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

aminoglycoside, glioma, hybrid nanovector, lentivirus, suicide gene

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