Efficient Inhibition of C-26 Colon Carcinoma by VSVMP Gene Delivered by Biodegradable Cationic Nanogel Derived from Polyethyleneimine

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ABSTRACT Biodegradable cationic nanoparticles have promising application as a gene delivery system. In this article, heparin—polyethyleneimine (HPEI) nanogels were prepared, and these nanogels were developed as a nonviral gene vector. The transfection efficiency of HPEI nanogels was comparable with that of PEI25K, while the cytotoxicity was lower than that of PEI2K and much lower than that of PEI25K *in vitro*. These HPEI nanogels also had better blood compatibility than PEI25K. After intravenous administration, HPEI nanogels degraded, and the degradation products were excreted through urine. The plasmid expressing vesicular stomatitis virus matrix protein (pVSVMP) could be efficiently transfected into C-26 colon carcinoma cells by HPEI nanogels *in vitro*, inhibiting the cell proliferation through apoptosis induction. Intraperitoneal injection of pVSVMP/HPEI complexes efficiently inhibited the abdominal metastases of C-26 colon carcinoma through apoptosis induction (mean tumor weight in mice treated with pVSVMP/HPEI complex = 0.93 g and in control mice = 3.28 g, difference = 2.35 g, 95% confidence interval [CI] = 1.75—2.95 g, $P < 0.001$) and prolonged the survival of treated mice. Moreover, intravenous application of pVSVMP/HPEI complexes also inhibited the growth of pulmonary metastases of C-26 colon carcinoma through apoptosis induction. The HPEI nanogels delivering pVSVMP have promising application in treating colon carcinoma.

KEYWORDS: nanotechnology · cancer · gene therapy · polyethyleneimine · vesicular stomatitis virus matrix protein

Cancer is a major public health problem in the world. Currently, 1 in 4 deaths in the United States is caused by cancer. Colon cancer has high incidence: more than 100 000 new colon cancer cases and about 50 000 deaths every year in the United States.[1,2] Gene therapy holds great promise for the treatment of various forms of diseases for which there is little hope of finding a conventional cure.[3] Initially, gene therapy was viewed as an approach for treating hereditary diseases; currently, its potential in cancer treatment is widely recognized.[4−6] The vesicular stomatitis virus matrix protein (VSVMP), one of the five structural proteins (N, P, M, G, and L) of this virus, can cause considerable cytopathogenesis of vesicular stomatitis virus (VSV) in the absence of other viral components. Matrix protein induced cytopathic effects through disruption of three types of cytoskeletal elements (actin, vimentin, and tubulin) by interacting with them and through general inhibition of host cell gene expression results in the systemic breakdown of the cell by apoptosis.[7−10] The VSVMP has received attention as an anticancer agent because of its ability to induce apoptosis.[11−14] This implies that there is potential to develop a gene therapy protocol for colon cancer based on the VSVMP gene.

In addition to target genes, the gene delivery system is very important when a gene therapy protocol is developed. Some functional genes associated with cancer have been discovered, but the clinical use of gene therapy treatments was restricted mainly because of the absence of safe and efficient gene delivery technologies. Although viruses have high transfection efficiency, it always induces side effects; this is a critical barrier.[15,16] After some failures of clinical gene therapy caused by severe side effects of viral vectors, safety becomes the first issue to be considered when the advanced gene delivery system is developed for clinical gene therapy. Nonviral gene carriers have advantages over viral vectors: the ability to deliver larger DNA molecules, low immunogenicity, relative safety, ease in production, and scaling up, *etc.* So, it is interesting to develop nonviral gene carriers.[17−20]

Because of electrostatic interaction, cationic polymers can bind and compact DNA; this means they may have potential application as gene carriers.[21−24] In addition to

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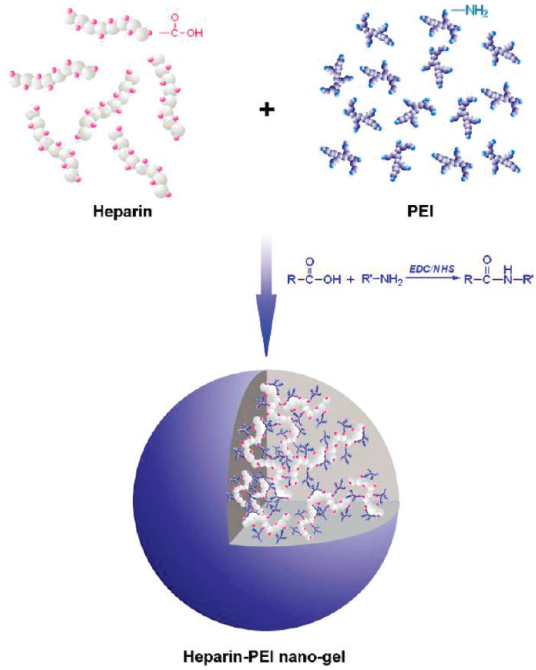
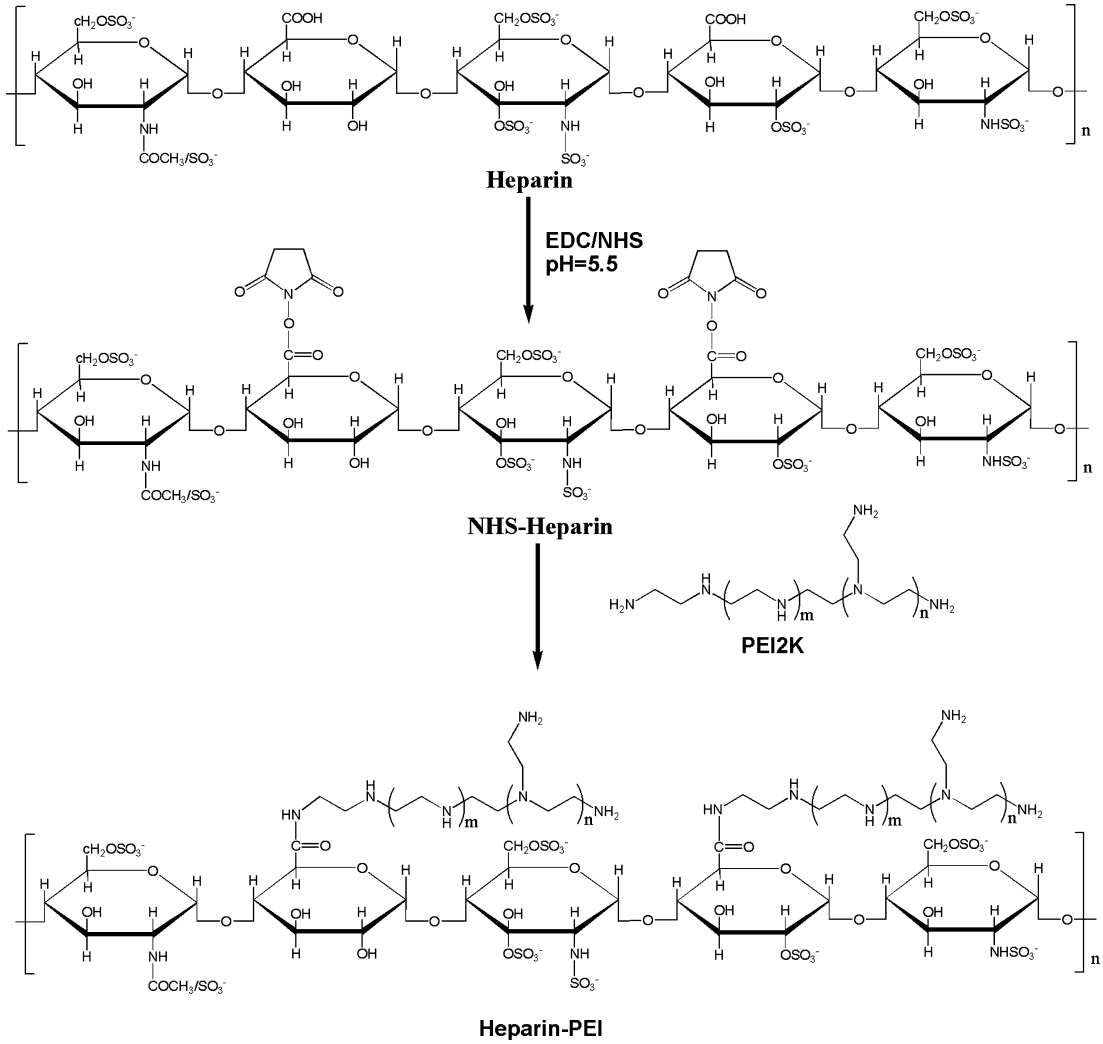


Scheme 1. Preparation scheme of heparin−PEI nanogel.

water-soluble polycations, cationic nanoparticles (derived from cationic polymers) can be used as gene vectors.[25−29] In 1995, cationic polyethyleneimine (PEI) was used as a gene carrier for the first time; currently, it has become one of the most efficient nonviral gene transfection agents.[30−32] The commercially available branched PEI (25 000 g/mol, PEI25K) has been widely used as a "gold standard" to evaluate transfection efficiency of other newly developed polymer- or surfactant-based gene carriers. However, PEI is not biodegradable and has a shortcoming; that is, the increase in transfection efficiency is accompanied by the increase in cytotoxicity, and both efficiency and cytotoxicity increase as its chain length increases.[33,34] One interesting approach to overcome this issue is to couple short PEI chains into a longer one using biodegradable linkers.[35−40] In this work, to develop a safe and efficient gene vector, PEI2K was chemically conjugated by heparin, resulting in biodegradable cationic nanogels. Then, the obtained heparin−PEI nanogels were employed to deliver pVSVMP to treat C-26 colon carcinoma *in vitro* and *in vivo*. The prepared HPEI nanogels could



Scheme 2. Chemical reaction between heparin and PEI.

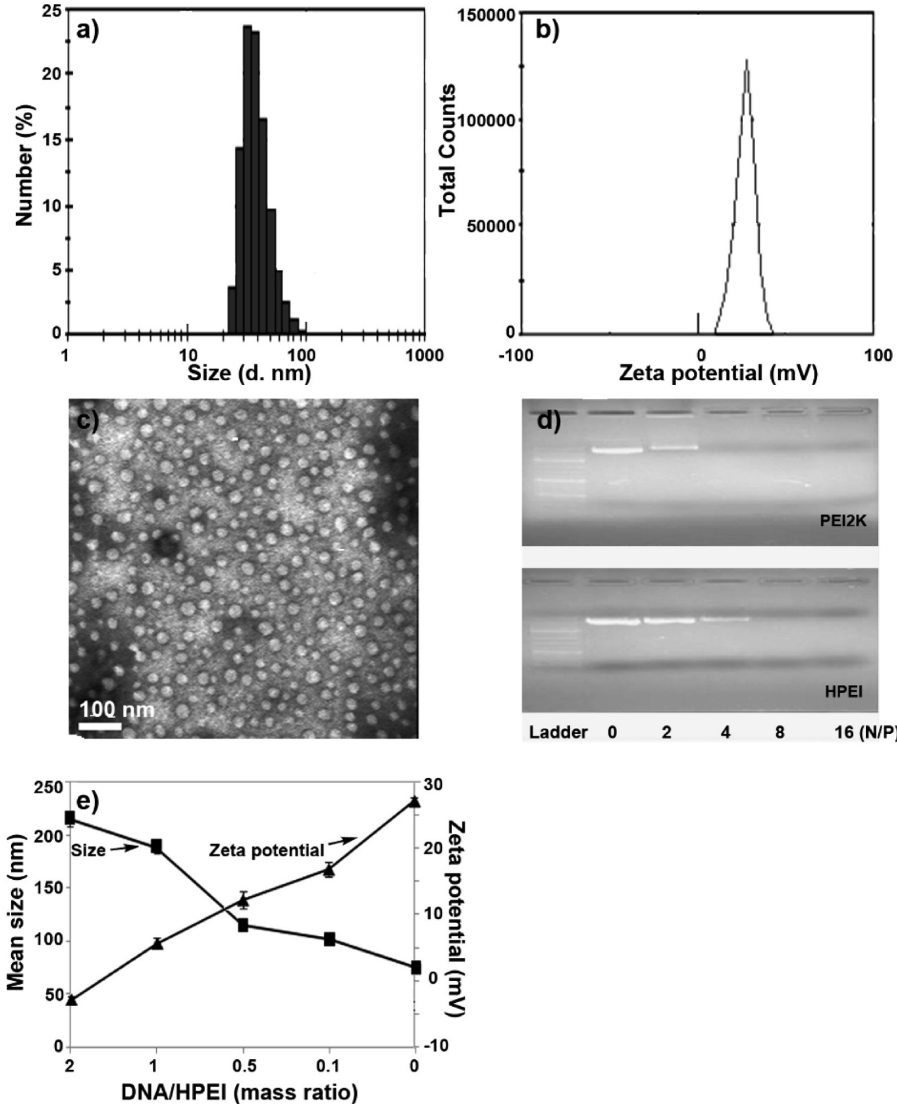


Figure 1. Characterization of HPEI nanogels. (a) Size distribution spectrum of HPEI nanogels; (b) zeta potential spectrum of HPEI nanogels; (c) TEM image of HPEI nanogels; (d) the DNA-binding ability of HPEI nanogels that determined by gel retardation assay; and (e) effect of DNA/HPEI mass ratio on the particle size and zeta potential of DNA/HPEI complexes.

be a novel nonviral gene vector, and pVSVMP delivered by HPEI nanogels could have potential application in colon carcinoma gene therapy.

RESULTS

Preparation and Characterization of HPEI Nanogels. To develop an efficient and safe gene vector, we prepared a biodegradable cationic nanogel: HPEI nanogels; it was derived from low molecular weight PEI. The preparation scheme of HPEI nanogel is presented in Scheme 1. Catalyzed by EDC/NHS, reaction between an amino group and a carboxyl group occurs, forming amine groups. Heparin is a biodegradable negative polysaccharide with many carboxylic groups in its molecular structure. PEI is a cationic polymer with many primary amine groups in its molecular structure. So, in presence of EDC/NHS, the reaction between heparin and PEI occurs. In our experiment, while consistently stirring, EDC/NHS activated heparin was dropped into PEI solution. This led to one heparin molecule reacting with several PEI molecules, also cross-linkage between heparin and PEI occurred; thus, through amide bond heparin conjugated PEI molecules, forming a HPEI nanogel. The composition has an important effect on the properties of HPEI nanogels. We also optimized the weight ratio of heparin and PEI. When the weight ratio of heparin and PEI was 1/1, the result was aggregation (not HPEI nanogels); thus this weight ratio was discarded. When the weight ratio of heparin and PEI was either 1/3 or 1/6, the products were HPEI nanogels; compared to a weight ratio of 1/6, the weight ratio of 1/3 led to the product with higher transfection efficiency. So a weight ratio of 1/3 was selected. The reaction equation between heparin and PEI is shown in Scheme 2. First, the carboxylic groups in heparin were activated by EDC/NHS, creating NHS−heparin. Then the reaction between NHS−heparin and PEI occurred, as the NHS−heparin tends to react with the primary groups

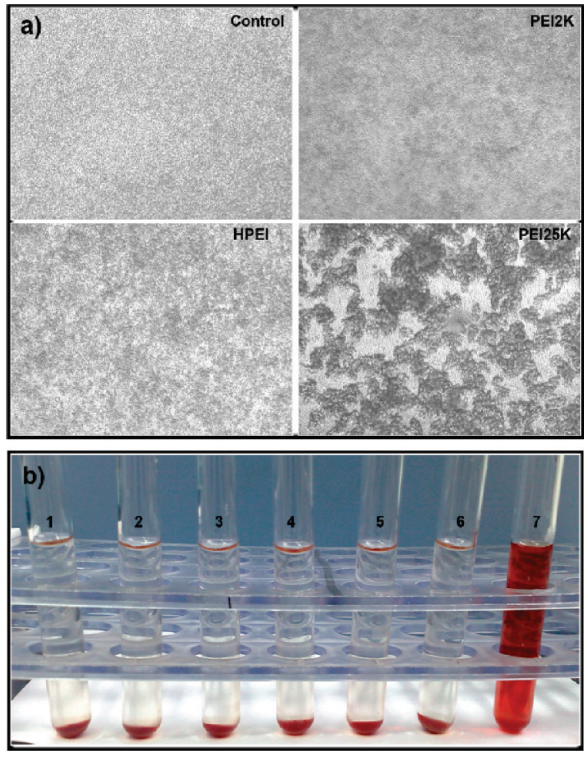


Figure 2. Evaluation of the blood compatibility of HPEI nanogels. (a) Microscopic images of erythrocyte aggregation induced by PEI2K, HPEI nanogels, and PEI25K. (b) Hemolytic test on the HPEI nanogels. The concentration of HPEI nanogels was 1 mg/mL (1), 0.8 mg/mL (2), 0.6 mg/mL (3), 0.4 mg/mL (4), or 0.2 mg/mL (5); sample 6 was normal saline used as negative control, and sample 7 was distilled water used as positive control.

in PEI. Both heparin and amide groups (formed by the reaction between carboxylic group in heparin and primary amine group in PEI) are biodegradable; thus, the HPEI nanogel is biodegradable.

The prepared HPEI nanogels were characterized in detail, and results are presented in Figure 1. The size distribution spectrum of HPEI nanogels is presented in Figure 1a; it indicates that HPEI nanogels were monodisperse (PDI = 0.157) and had a mean particle size of 75 ± 6.6 nm. The zeta potential spectrum of HPEI nanogels is presented in Figure 1b; these HPEI nanogels were cationic and had the zeta potential of +27 ± 0.71 mV. Moreover, the TEM image of HPEI nanogels (shown in Figure 1c) indicated that these HPEI nanogels were monodisperse and had the mean particle size of ~25 nm. TEM determines the size of dry particles, while the dynamic light scattering determines the hydrodynamic diameter of particles in water. These HPEI nanogels likely have high water absorption, as dry HPEI nanoparticles (~25 nm) can absorb water and swell to become nanogels with a size of ~75 nm. Conventionally, anionic heparin and cationic PEI form a polyionic complex due to electrostatic interaction, but according to our preliminary experiments, those polyionic complexes had difficulty transfecting pDNA into cells *in vitro* and also tended to form aggregates after dialysis. Catalyzed by EDC/NHS, reaction between the carboxylic group and the primary amine occurs easily, forming an amide group; thus, PEI can be easily conjugated by heparin through the amide group in the presence of EDC/NHS. Moreover, the described HPEI nanogels efficiently transfected pDNA into cells and were stable after dialysis. According to the TEM image, monodisperse nanoparticles also were evident. So, this suggests that the previously described HPEI nanogels probably are heparin−PEI covalent conjugates efficiently compacted due to the formation of intramolecular polyionic complexes with an overall positive charge.

The small size and positive charge of HPEI nanogels encouraged us to deeply understand its potential application as a nonviral gene vector. The DNA-binding ability of the HPEI nanogels was evaluated by gel retardation assay (shown in Figure 1d). Both PEI2K and HPEI nanogels could efficiently bind DNA. At the ratio of nitrogen atoms to phosphate group PEI2K (N/P) of 4, the complete retardation of DNA was achieved; for the phosphate group HPEI, however, the retardation of DNA was achieved at level 8 (N/P). Compared with PEI2K, the decreased DNA-binding ability of HPEI nanogels may be mainly due to the loss of primary amine groups caused by the reaction between PEI and heparin.

HPEI nanogels are cationic, while pDNA is anionic. Due to electrostatic interaction, pDNA can be absorbed on the surface of HPEI nanogels, forming a pDNA/HPEI complex, thus resulting in pDNA encapsulation. According to our previous studies, binding plasmid to the surface of a cationic nanoparticle efficiently protects plasmid from enzymatic degradation and acidic degradation for a certain time.[41] Thus, binding DNA to cationic HPEI nanogels may result in a certain degree of protecting DNA from enzymatic degradation and acidic degradation. Moreover, the effects of DNA/HPEI mass ratio on the size and zeta potential of DNA/HPEI complexes were studied (shown in Figure 1e): with a decrease in DNA/HPEI mass ratio, the particle size decreased and zeta potential increased.

The erythrocyte aggregation and hemolysis analyses were used to investigate the blood compatibility of HPEI nanogels. These results are presented in Figure 2. Figure 2a demonstrates that PEI25K treatment induced obvious aggregation of erythrocytes, but HPEI nanogels and PEI2K did not induce severe erythrocyte aggregation. As shown in Figure 2b, HPEI nanogel did not induce hemolysis in our experiment. Figure 2 shows that the HPEI nanogel might have better blood compatibility than PEI25K. Otherwise, intravenous administration of PEI25K at the dose of 30 mg/kg caused rat death, but intravenous application of 60 mg/kg of HPEI nanogels did not lead to rat death. Thus, HPEI nanogel has lower toxicity than PEI25K *in vivo*.

The biodegradability of HPEI nanogels was also evaluated on rats *in vivo*. Three milligrams of PEI2K,

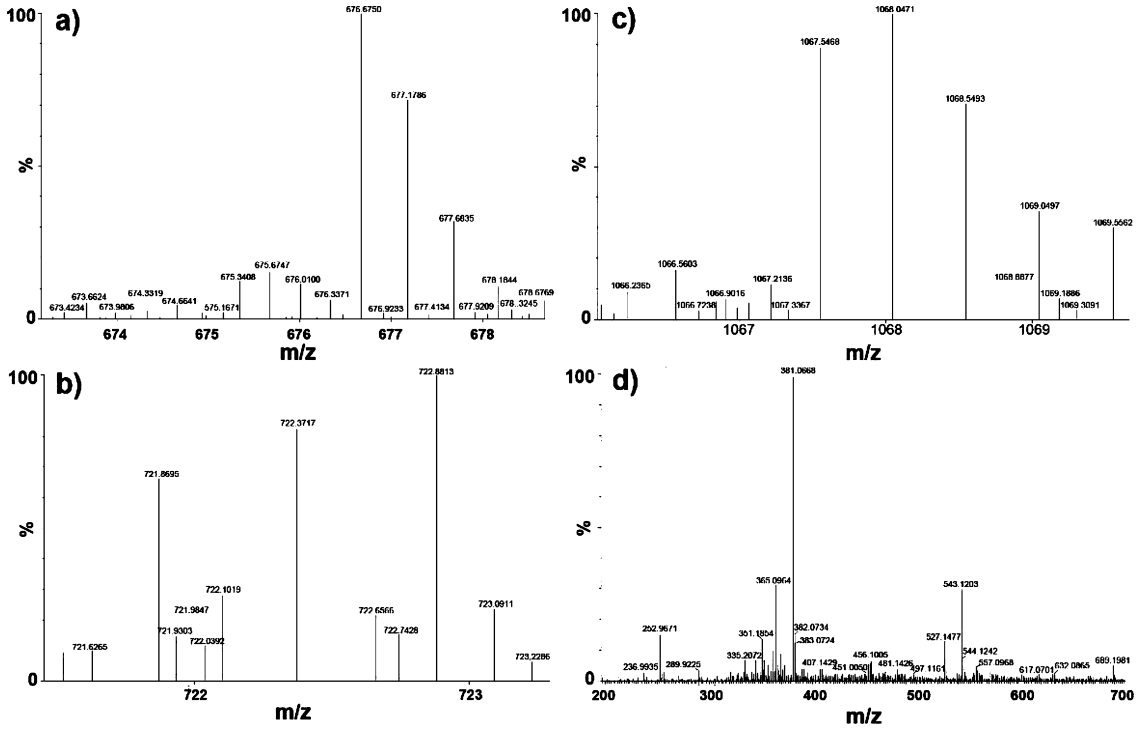


Figure 3. Mass spectra qualitatively determined the degradation process of HPEI nanogels after i.v. application: (a) mass spectrum of standard PEI2K, (b) mass spectrum of the extraction of urine from rat treated with HPEI nanogels, (c) mass spectrum of the extraction of urine from rat treated with PEI2K, and (d) mass spectrum of the extraction of urine from rat treated with PEI25K.

HPEI nanogels, or PEI25K was intravenously injected into rats; in the following 24 h, the PEI in the urine or feces of rats was qualitatively determined by mass spectrometer. As determined by mass spacing of PEI2K, as shown in Figure 3a, the *m/z* range was mainly composed of doubly and triply charged species. In Figure 3b (HPEI nanogels treatment) and Figure 3c (PEI2K treatment), the *m/z* range was also composed of doubly and triply charged species; this may imply the presence of PEI in urine. However, in Figure 3d (PEI25K treatment), the *m/z* range is only composed of singly charged species; thus there was no PEI in the urine. Otherwise, PEI was not detected in the feces of all treatment groups (data not shown). So, this suggests that the HPEI nanogels can be quickly degraded into low molecular PEI, and the degradation products are excreted from the body through urine. Conventionally, the amide group is difficult to be quickly degraded in 24 h *in vivo*. In contrast, heparin degrades quickly *in vivo* because of the presence of enzymes. Thus, the quick degradation of HPEI nanogels may be due to the quick degradation of heparin (not amide groups) *in vivo*.

The transfection efficiency and cytotoxicity of HPEI nanogels were investigated *in vitro*, and results are presented in Figure 4. As shown in Figure 4a, both HEK293 and C-26 cells can be transfected by the pGFP/HPEI complex. The transfection efficiency of pGFP/HPEI complex on HEK293 or C-26 cells was 39.4 ± 3.8 or 27.6 ± 2.6%, respectively, while PEI25K (as a standard transfection agent) transfected pGFP into HEK-293 or C-26 cells

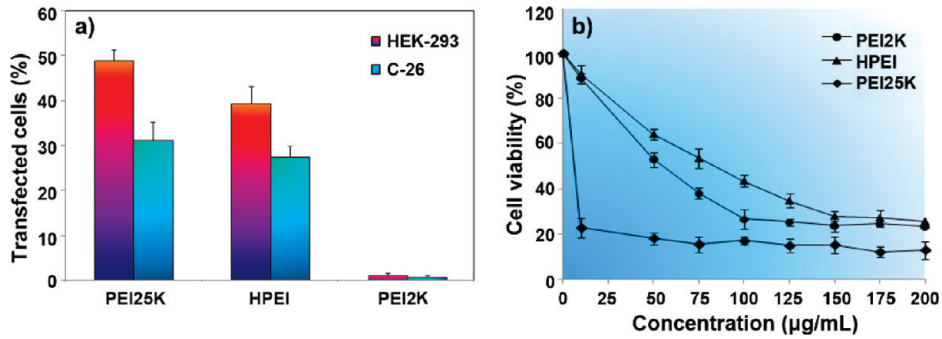


Figure 4. Transfection efficiency and cytotoxicity of HPEI nanogels. (a) Transfection efficiency of PEI25K, PEI2K, and HPEI nanogels on HEK-293 cell line and C-26 cell line. The amount of pGFP was kept at 2 μg/well, while the mass ratio of HPEI/pGFP, PEI25K/pGFP, or PEI2K/pGFP was 5/1, 1/1, or 5/1, respectively. Flow cytometry (Epics Elite ESP, USA) was used to determine the % transfected cells. (b) Cytotoxicity of PEI25K, PEI2K, and HPEI nanogels on C-26 cells.

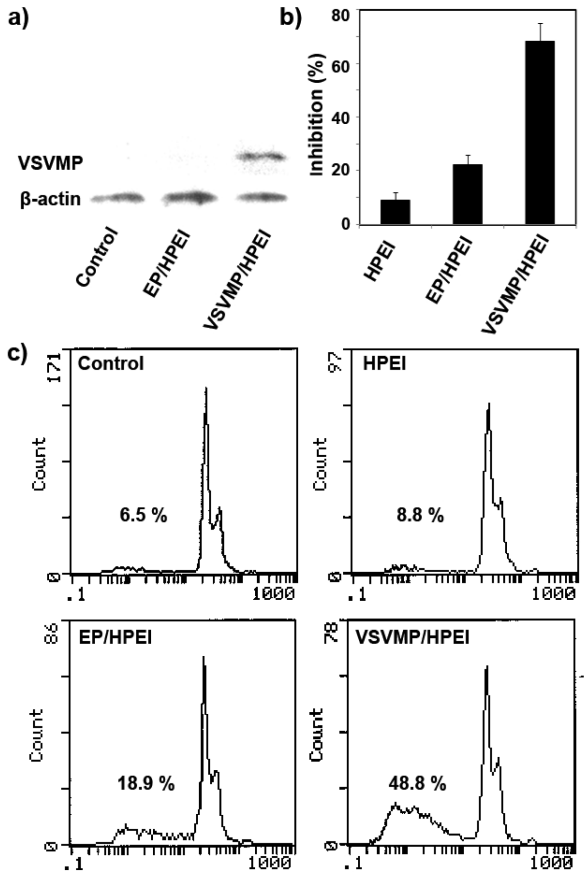


Figure 5. HPEI nanogels transfected pVSVMP into C-26 cells, inhibiting the growth of C-26 cells *via* apoptosis induction *in vitro*. (a) After transfection with pVSVMP/HPEI complexes for 48 h, C-26 cells expressed VSVMP (analyzed by Western blotting assay). (b) Growth inhibition of C-26 cells treated by HPEI nanogels (HPEI), pEP/HPEI complexes (EP/HPEI), or pVSVMP/HPEI complexes (VSVMP/HPEI). (c) DNA fluorescence histograms of propidium iodide stained C-26 cells treated with normal saline (control), HPEI nanogels (10 μg), pEP/HPEI complexes (2 μg/10 μg), or pVSVMP/HPEI complexes (2 μg/10 μg) for 48 h.

with the transfection efficiency of 49.1 ± 2.3 or 31.3 ± 4.1%, respectively. The transfection efficiency of HPEI nanogels on the C-26 cell line is comparable to that of PEI25K ($p > 0.05$). From results presented in Figure 4b, it is evident that the PEI25K is very toxic, and the IC_{50} was <10 μg/mL. The HPEI nanogels with an IC_{50} of 77.04 μg/mL had lower toxicity than PEI2K (IC_{50} = 55.5 μg/mL); this may be due to the presence of nontoxic heparin in HPEI nanogels and the reduced content of primary amine groups. The transfection efficiency studies and cytotoxicity analysis imply that the HPEI nanogel is a novel gene vector with high transfection efficiency and low toxicity.

Moreover, the stability of HPEI nanogels also was evaluated *in vitro*. After storage for 2 months at 4 °C, HPEI nanogels were monodisperse (PDI = 0.169) and had the mean particle size of 79 ± 5.6 nm, as determined by dynamic light scattering. The transfection efficiency of HPEI nanogels also was maintained after storage (data not shown). This implies that HPEI nanogels have good stability *in vitro*.

Anticancer Activity of pVSVMP/HPEI Complexes on C-26 Cells *In Vitro*. The anticancer activity of pVSVMP/HPEI complexes on the C-26 cell line was evaluated *in vitro*. After transfection with the pVSVMP/PEI complex for 48 h, VSVMP was detected in the C-26 cell by Western blot assay, shown in Figure 5a; this implies that HPEI nanogels efficiently transfected pVSVMP into C-26 cells. From Figure 5b, pVSVMP/HPEI complexes (2 μg DNA/10 μg HPEI), pEP/HPEI complexes (2 μg DNA/10 μg HPEI), and HPEI nanogels (10 μg) caused 68.3, 22.4, and 9.1% inhibition of C-26 cell growth, respectively. To evaluate whether the antiproliferative effect of pVSVMP/HPEI complexes was associated with apoptosis induction, we employed flow cytometry to detect apoptotic cells, finding that the apoptotic cells (sub-G1 cells) among C-26 cells accounted for 48.8, 18.9, 8.8, and 6.5% when treated with pVSVMP/HPEI complexes, pEP/HPEI complexes, HPEI nanogels, and normal saline, respectively. Thus, pVSVMP/HPEI complexes efficiently induced the apoptosis of C-26 cells. So, it is suggested that HPEI nanogels can efficiently transfect pVSVMP into C-26 colon carcinoma cells *in vitro*, thus inhibiting cell proliferation through apoptosis induction.

Anticancer Effect of pVSVMP/HPEI Complexes on C-26 Cell Line *In Vivo*. The anticancer activity of the intraperitoneal injection of pVSVMP/HPEI complexes on the abdominal cavity metastases of C-26 colon carcinoma is illustrated in Figure 6. Figure 6a shows representative images of abdominal cavity metastases of C-26 colon carcinoma in each treatment group. It is obvious that the mice treated with pVSVMP/HPEI complexes bore fewer abdominal cavity metastases than other groups. The metastases in each group were harvested and weighed. The weight of the tumors in each group is presented in Figure 6b. The T_w/C_w, the ratio of the mean weight of abdominal cavity metastases of C-26 colon carcinomas in treated mice (T_w) divided by that of the control group (C_w), was 0.63 (mean 2.07 *versus* 3.28 g, difference = 1.21 g, 95% CI = 0.61 to 1.81 g, $P < 0.01$) in the pEP/HPEI complex treatment group and 0.28 (mean 0.93 *versus* 3.28 g, difference = 2.35 g, 95% CI = 1.75 to 2.95 g, $P < 0.01$) in pVSVMP/HPEI complex treated mice. Compared with pEP/HPEI complexes, pVSVMP/HPEI complexes caused a statistically significant reduction in tumor weight (mean 0.93 *versus* 2.07 g, difference = 1.14 g, 95% CI = 0.54 to 1.74 g, $P < 0.01$). Thus, tumor growth in mice with pVSVMP/HPEI complex treatment was dramatically suppressed. As shown in Figure 6c, there is a statistically significant increase in the life span of the pVSVMP/HPEI complex treated mice, compared with that of other groups. The life span of mice with pVSVMP/HPEI complex treatment was 39.8 days (95% CI = 36.9 to 41.2 days) compared with 20.6 days (95% CI =16.7 to 25.3 days) for mice in the control group, 21 days (95% CI = 17.9 to 22.2 days) for mice treated with HPEI nanogels, and 26.4 days (95% CI = 23.9 to 28.2 days) for mice treated with pEP/HPEI complexes. Com-

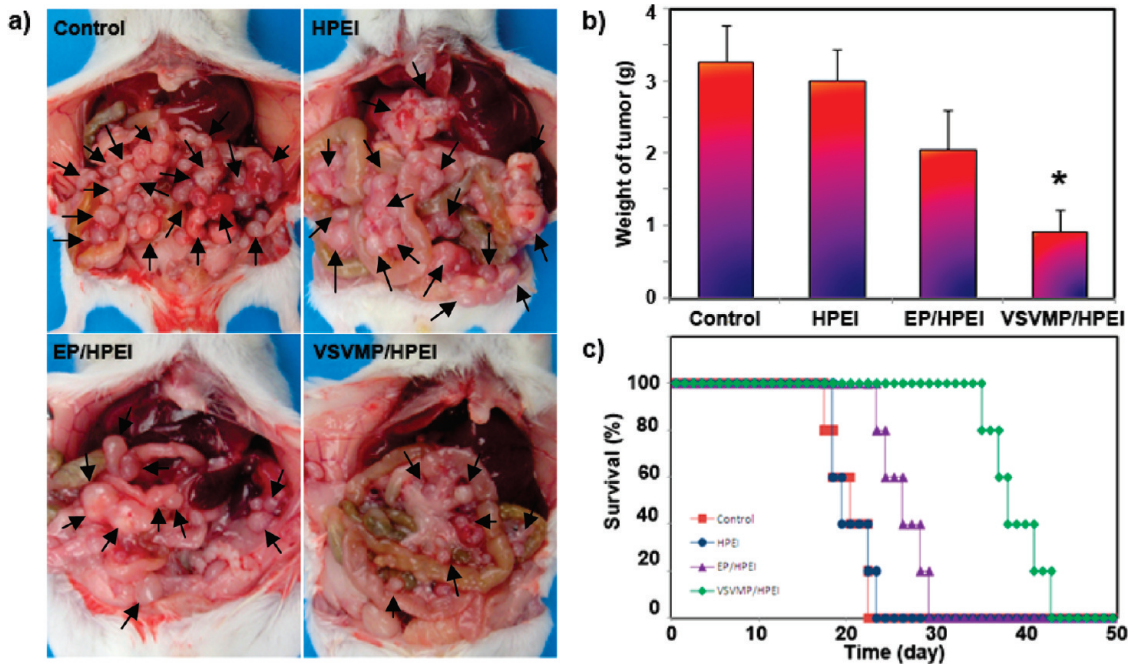


Figure 6. Intraperitoneal administration of pVSVMP/HPEI complexes inhibited the growth of abdominal metastases of C-26 colon carcinoma and prolonged the survival of treated mice. (a) Representative photographs of abdominal metastatic nodes (arrows) in each treatment group. (b) Weight of abdominal metastases of C-26 colon carcinoma in each treatment group. (c) Survival curves of mice in each treatment group.

pared to pEP/HPEI complex treatment, pVSVMP/HPEI complexes more efficiently prolonged the survival of mice bearing abdominal cavity metastases of C-26 colon carcinoma ($P < 0.01$). These results suggest that intraperitoneal injection of pVSVMP/HPEI complexes can efficiently inhibit the growth of abdominal cavity metastases of C-26 colon carcinoma and prolong the survival of mice.

To study the mechanism associated with the anticancer activity of pVSVMP/HPEI complexes *in vivo*, TUNEL assay was carried out. As shown in Figure 7, many strongly positive nuclei identified as apoptosis could be observed in the pVSVMP/HPEI complex treated tumor tissue, whereas such nuclei were rare in other groups. This implies that apoptosis induction may be an important mechanism of inhibiting colon cancer by the pVSVMP/HPEI complex *in vivo*.

For the pulmonary metastatic C-26 colon carcinoma model, after intravenous application of 10 doses of pVSVMP/HPEI complexes, the mice were killed on

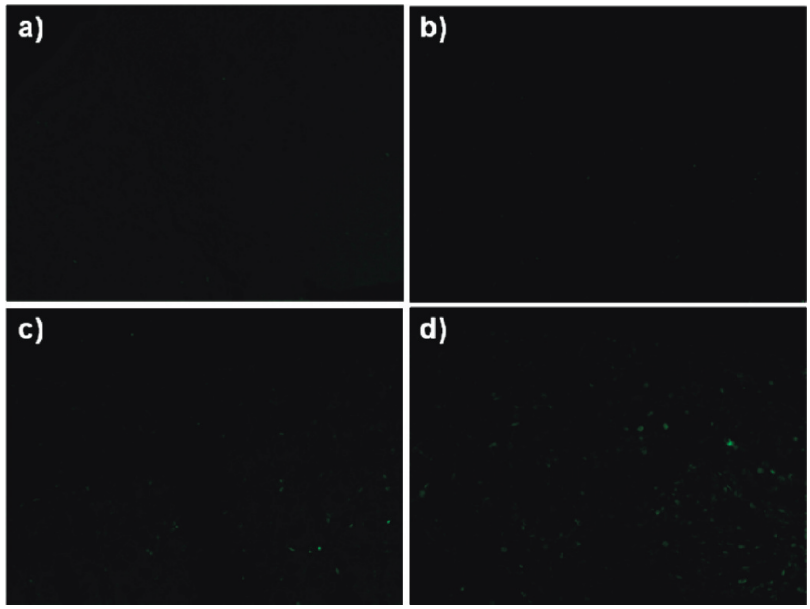


Figure 7. TUNEL staining of abdominal metastases of C-26 colon carcinoma in (a) control group, (b) HPEI nanogel treatment group, (c) pEP/HPEI complex treatment group, or (d) pVSVMP/HPEI complex treatment group.

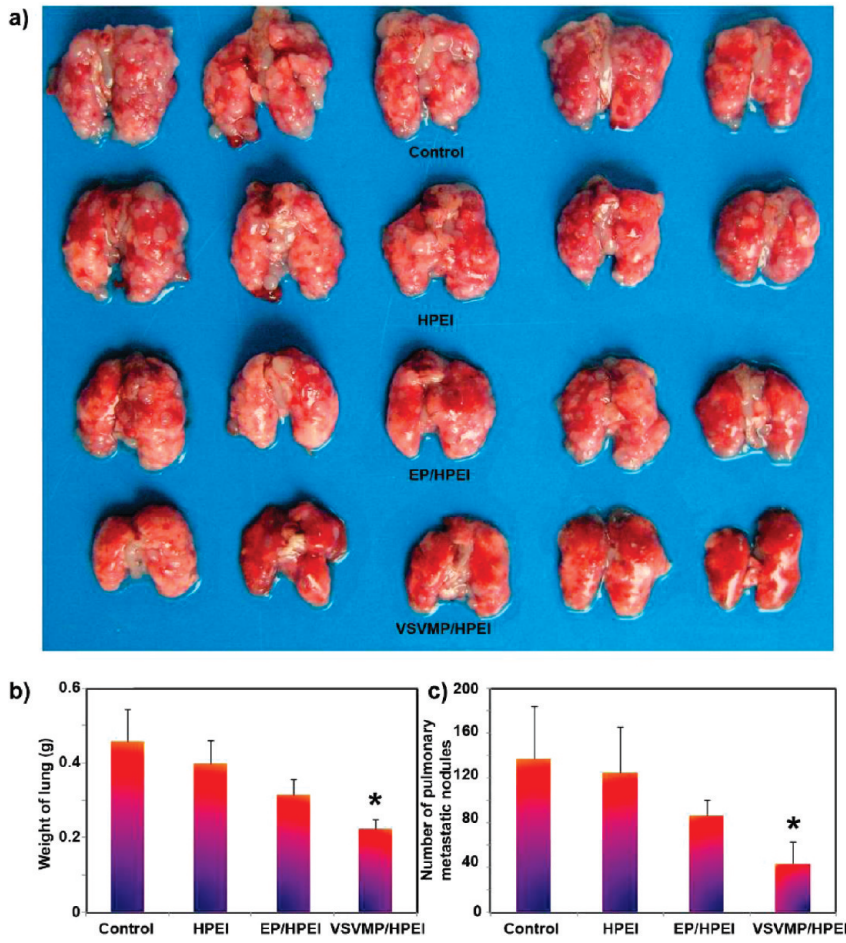


Figure 8. Intravenous application of pVSVMP/HPEI complexes inhibited the growth of pulmonary metastases of C-26 colon carcinoma. (a) Photograph of lungs bearing metastases of C-26 colon carcinomas in each treatment group. (b) Weight of lungs bearing pulmonary metastases of C-26 colon carcinoma in each treatment group. Intravenous administration of pVSVMP/HPEI complexes efficiently inhibited the pulmonary metastases of C-26 colon carcinoma, reducing the weight of lung ($P < 0.01$ *vs* control; $P < 0.01$ *vs* HPEI; $P < 0.05$ *vs* EP/HPEI). (c) Number of pulmonary metastatic nodules in each treatment group. Mice in the pVSVMP/HPEI treatment group bear less pulmonary metastatic tumor nodules than other groups ($P < 0.01$ *vs* control; $P < 0.01$ *vs* HPEI; $P < 0.05$ *vs* EP/HPEI).

day 18. The lungs were harvested, weighed, and analyzed. From Figure 8a, it can be seen that lungs of mice with pVSVMP/HPEI complex treatment bore less pulmonary metastatic nodules than those of mice in other groups. The inhibitory effect on metastases of pVSVMP/HPEI complexes was reflected in a statistically significant reduction in weight of the lung compared to other groups (mean for mice treated with pVSVMP/HPEI complexes = 0.23 g *versus* 0.46 g in controls, difference = 0.23 g, 95% CI = 0.13 to 0.31, $P < 0.01$; *versus* 0.40 g in HPEI nanogel treatment group, difference = 0.17 g, 95% CI = 0.09 to 0.27 g, $P < 0.01$; *versus* 0.32 in pEP/HPEI complex treatment group, difference = 0.09 g, 95% CI = 0.01 to 0.19 g, $P < 0.05$), shown in Figure 8b. Moreover, the pulmonary metastatic nodules in each mouse were numbered, and results are presented in Figure 8c. The metastatic nodules in the pVSVMP/HPEI complex treated group were less than that in other groups (mean number of metastatic nodules in pVSVMP/HPEI complex treatment group = 43 *versus* 137 in controls, difference = 94, 95% CI = 49.07 to 139.33, $P < 0.01$; *versus* 125 in HPEI nanogels treatment group, difference = 82, 95% CI = 36.27 to 126.53, $P < 0.01$; *versus* 88 in pEP/HPEI complexes treatment group, difference = 45, 95% CI = 0.67 to 90.93, $P < 0.05$). This indicates that intravenous application of pVSVMP/HPEI complexes can efficiently inhibit the growth of pulmonary metastases of C-26 colon carcinoma *in vivo*.

In the above two models, gross changes such as weight loss, ruffling of fur, and changes in behavior were not seen in pVSVMP/HPEI complex treated mice. In addition, no obvious pathological changes of heart, liver, kidney, lung, spleen, or brain were found by microscopic examination.

DISCUSSION

In this paper, we develop a novel gene therapy protocol for colon carcinoma. A gene therapy protocol is always composed of two components: target gene and gene delivery system. Cationic nanoparticles show promising application as a gene delivery system. First,

biodegradable cationic HPEI nanogels, as a new gene carrier, were prepared and characterized in detail. Then, this carrier was used to deliver pVSVMP to treat C-26 colon carcinoma *in vitro* and *in vivo*. Our results indicated that HPEI nanogels could be a novel nonviral gene vector, and HPEI nanogels delivering pVSVMP had promising application in colon carcinoma therapy.

Polyethyleneimine (PEI) is proven to be effective in gene delivery due to its condensation of DNA, which facilitates endocytosis, as well as its "proton sponger" quality, which can prevent the DNA from endosomal disruption.[30−32] However, PEI is not biodegradable and has a shortcoming; that is, the improvement of transfection efficiency is accompanied by increased cytotoxicity, and both efficiency and cytotoxicity increase with its chain length. Efficacy and adverse reactions of PEI thereby seem to be strongly associated. To break up the correlation between efficacy and toxicity of PEI as a nonviral gene vector, other scientists have coupled short PEI chains into a longer one using biodegradable linkers, increasing the transfection efficiency and reducing the toxicity of PEI. Previously, some linkers had been used to conjugate low molecular weight PEI, among which the most studied linkers were PEG, Pluronic, PLA, PCL, *etc.*[42−45] In these linkers, PEG and Pluronic have good biocompatibility but are not degradable; PLA and PCL are degradable due to hydrolysis of the ester bond, but before application, these PEI derivatives degrade quickly (because the PEI derivatives' aqueous solution is always basic, and an ester bond tends to quickly degrade in basic solution). Contrary to the previously described *in vitro* quick degradation process, in physiological conditions, the degradation of the ester bond is slow; thus, another shortcoming of those polymers is due to their ester linkages as cross-linkers, proving an undesirably long half-life *in vivo*. Because of the following considerations, in this work, low molecular weight PEI was chemically conjugated into biodegradable cationic nanogels by heparin. First, heparin (a natural polysaccharide) is biocompatible and nontoxic. Second, heparin is relatively stable *in vitro* and can be degraded due to hydrolysis and enzymolysis *in vivo*. Third, heparin has many carboxylic groups in its molecular composition. Catalyzed by EDC/NHS, reaction between carboxylic group in heparin and primary amine in PEI forms an amide group easily. The amide group is stable in weak basic solution and can be degraded *in vivo*. Fourth, introducing heparin into biomaterials improves the biocompatibility.[46,47]

The obtained monodisperse cationic HPEI nanogels had the hydrodynamic diameter of 75 nm, showed DNA-binding ability, and could efficiently transfect genes into cells. The transfection efficiency of HPEI nanogels was comparable to PEI25K on C-26 cells. Moreover, the cytotoxicity of HPEI nanogels was lower than that of PEI2K and much lower than that of PEI25K. HPEI nanogels also showed better blood compatibility than PEI25K. Furthermore, the HPEI nanogels were stable *in vitro* and could be quickly degraded into low molecular weight PEI followed by excretion through urine. These features of HPEI nanogels suggest that HPEI nanogels could be a good gene carrier and have potential clinical application.

After HPEI nanogels were obtained, they were used to deliver pVSVMP to treat C-26 colon carcinoma *in vitro* and *in vivo*. Vesicular stomatitis virus (VSV), the prototype virus of the Rhabdoviridae, can preferentially replicate in cancer cells and finally induce apoptosis. The growth-inhibiting effect of VSV has been demonstrated in various tumor models.[48,49] However, the application of VSV is limited by the potential biohazard of virus infection. The VSVMP, one of the five structural proteins (N, P, M, G, and L) of this virus, can inhibit host cell gene expression, resulting in the systemic breakdown of the cell through apoptosis.[8−10] So, VSVMP, but not VSV, can be used to treat cancers. Recently, some cancer gene therapy protocols based on pVSVMP have been developed; these research results were promising.[11−14] In some protocols, cationic liposome had been used as a nonviral gene vector to deliver pVSVMP, but these shortcomings of cationic liposome (such as high cost, strong cytotoxicity, low transfection efficiency *in vivo*, *etc.*) restricted its clinical application. Colon carcinoma has high incidence and mortality rate. To develop an advanced gene therapy protocol for colon carcinoma, in this paper, pVSVMP was delivered by the HPEI nanogel to treat colon carcinoma *in vitro* and *in vivo*. In our experiments, HPEI nanogels efficiently transfected pVSVMP into C-26 cells, inhibiting the proliferation of C-26 cells through apoptosis induction *in vitro*. Meanwhile, pVSVMP delivered by the HPEI nanogel significantly inhibited abdominal metastases and pulmonary metastases of C-26 colon carcinoma through apoptosis induction *in vivo*. Because of the anticancer activity, administration of pVSVMP/HPEI complexes also prolonged the survival of mice bearing C-26 colon carcinoma. These results suggest that pVSVMP delivered by HPEI nanogels may have promising clinical application in C-26 colon carcinoma therapy.

Recently, Jeon *et al.* synthesized water-soluble heparin−PEI conjugates and studied the potential application of cationic heparin−PEI conjugates for gene delivery.[50] According to their study, heparin conjugation to PEI could improve the *in vivo* gene transfection efficiency of PEI. Just as water-soluble polycations, cationic nanoparticles also have potential application as a gene delivery system. In this work, due to the different experimental parameters from those reported by Jeon *et al.*, HPEI nanogels, and not a water-soluble polymer, were prepared. Although Jeon's heparin−PEI conjugates and our HPEI nanogels are both made from heparin and PEI, their structures are different. Meanwhile, Jeon's heparin−PEI conjugates were used to deliver pVEGF to induce neovascularization, but our HPEI

nanogels were employed to deliver anticancer gene (pVSVMP) to treat cancer (C-26 colon carcinoma). To our knowledge, this might be the first report on HPEI nanogels for anticancer gene delivery; thus, treating colon cancer by HPEI nanogels delivered pVSVMP may be a new and interesting cancer gene therapy protocol.

CONCLUSIONS

In this research, heparin−polyethyleneimine (HPEI) nanogel was prepared and used to deliver pVSVMP (plasmid expressing vesicular stomatitis virus matrix protein) to treat C-26 colon carcinoma *in vitro* and *in vivo*. These HPEI nanogels are a novel nonviral gene vector. HPEI nanogels can efficiently transfect pVSVMP into C-26 colon carcinoma cells *in vitro*, inhibiting cell proliferation through apoptosis induction. Application of pVSVMP/HPEI complexes can efficiently inhibit the growth of colon carcinoma *in vivo*. Treating colon cancer by the HPEI nanogel delivered pVSVMP may be a new and interesting cancer gene therapy protocol.

METHODS

Materials. Heparin (M_w = 4000−6000) was purchased from Fluka (USA). Methanol was purchased from KeLong Chemicals (Chengdu, China). Polyethyleneimine (M_w = 2000, PEI2K), polyethyleneimine (M_w = 25 000, PEI25K), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), MES, Dulbecco's modified Eagle's medium (DMEM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (USA).

Sprague−Dawley (SD) rats (weight of 200 ± 20 g) were purchased from the Laboratory Animal Center of Sichuan University (Chengdu, China) and used for evaluation of the biodegradability of HPEI nanogels *in vivo*. BALB/c mice (6−8 weeks old) were also purchased from the Laboratory Animal Center of Sichuan University and used to evaluate the anticancer effect of pVSVMP/HPEI complexes *in vivo*. The animals were housed at a temperature of 20−22 °C, relative humidity of 50−60%, with 12 h light−dark cycles. They were also provided with free access to food and water. All of the animals were in quarantine for a week before treatment. All animal care and experimental procedures were conducted according to Institutional Animal Care and Use guidelines.

Synthesis of HPEI Nanogels. Heparin−polyethyleneimine (HPEI) nanogels were prepared using amide bond formation between the amine groups of branched PEI and the carboxyl groups of heparin. Briefly, 50 mg of heparin was dissolved in a MES buffer solution (100 mL, 0.05 M); then, 20 mg of EDC and 30 mg of NHS were added into the above solution to activate the carboxylic acid groups of heparin. After 2 h of reaction at room temperature, this solution was dropped into 20 mL of PEI2K solution (7.5 mg/mL) while stirring consistently. The reaction was carried out at room temperature overnight. Later, the resultant HPEI nanogels were dialyzed (MWCO = 8000−14 000) in distilled water for 3 days. Then, the HPEI nanogels were filtered by a syringe filter (pore size = 220 nm) (Millex-LG, Millipore Co., USA). At last, the HPEI nanogels were adjusted to 1 mg/mL and stored at 4 °C for future use.

Preparation and Purification of pVSVMP. The pcDNA3.1 plasmid (Invitrogen, San Diego, CA) expressing wild-type VSVMP (named pVSVMP) was constructed in our laboratory as in our previous reports.[12−14] As a control, pcDNA3.1 plasmid without VSVMP-cDNA was used as an empty vector (named pEP). Colonies of *Escherichia coli* containing pVSVMP or pEP were cultured in Luria−Bertani broth containing ampicillin (100 μg/mL). Large-scale plasmid DNA was purified using an EndoFree Plasmid Giga kit (Qiagen, Chatsworth, CA). The DNA was eventually dissolved in sterile endotoxin-free water and stored at −20 °C before use. The recombinant pVSVMP was confirmed by restriction digestion and DNA sequencing.

Characterization of HPEI Nanogels. The particle size and zeta potential of HPEI nanogel or pVSVMP/HPEI complex were determined by dynamic light scattering (Malvern Nano-ZS 90) after equilibration for 5 min. All results were the mean of three test runs.

The morphology of HPEI nanogels was observed under a transmission electron microscope (TEM) (H-6009IV, Hitachi, Japan): nanogels were placed on a copper grid covered with nitrocellulose. The sample was negatively stained with phosphotungstic acid and dried at room temperature.

Gel Retardation Assay. The DNA/HPEI complexes were electrophoresed on 1% (w/v) agarose gel for 30 min at 100 V. The gel was stained with ethidium bromide (0.5 mg/mL) and illuminated on a UV illuminator to show the location of DNA. One microgram of pGFP plasmid was mixed with various amounts of HPEI nanogels.

Aggregate of Erythrocytes *In Vitro*. Fresh blood from Sprague−Dawley rat was collected in heparinized tubes. This blood was washed several times with normal saline until the supernatant was colorless. A 200 μL tube of erythrocytes (2%) was treated with 5 μg of samples (PEI2K, HPEI nanogels or PEI25K) in 200 μL of normal saline in 24-well plates. After it was incubated for 2 h at 37 °C, pictures of erythrocytes were taken with an optical microscope.

Hemolytic Test of HPEI Nanogels *In Vitro*. The hemolytic test of HPEI nanogels was studied *in vitro*. Briefly, 0.5 mL of HPEI nanogel with different concentrations in normal saline was diluted into 2.5 mL by normal saline, and this solution was added into 2.5 mL of rabbit erythrocyte suspension (2%) in normal saline at 37 °C. Normal saline and distilled water were employed as negative and positive control, respectively. Three hours later, the erythrocyte suspension was centrifuged (3000 rpm × 5 min), and the color of the supernatant was compared with the negative control. Absolute achromatic supernatant solution implies that there is no hemolysis. In contrast, red supernatant solution means hemolysis.

Biodegradability Study *In Vivo*. Rats were used to evaluate the biodegradability of HPEI nanogels *in vivo*. First, 3 mg of HPEI nanogels, PEI2K, or PEI25K was intravenously injected into SD rat. The urine and feces of rats were collected by metabolism cages in the following 24 h and were extracted by methanol. The resultant products were analyzed by a Quatrro Premier XE triple quadruple mass spectrometer (Micromass, UK).

***In Vitro* Gene Transfection.** Twenty four hours prior to transfection, HEK293 or C-26 cells were seeded into a 6-well plate (Becton-Dickinson, USA) at a density of 2×10^5 cells per well in 2 mL of complete medium (DMEM containing 10% FCS). At the time of transfection, the medium in each well was replaced with 1 mL of fresh serum-free medium. The pGFP was used as a report gene. The amount of pGFP was kept at 2 μg/well, while the mass ratio of HPEI/pGFP, PEI25K/pGFP, or PEI2K/pGFP was 5/1, 1/1, or 5/1, respectively. Six hours later, the medium was replaced by a complete medium. The transfection efficiency was recorded by flow cytometry (Epics Elite ESP, USA) after 24 h.

Cytotoxicity Assays. The cytotoxicity of HPEI nanogels on the C-26 cell line was evaluated by cell viability assay. Briefly, C-26 cells were plated at a density of 5×10^3 cells per well in 100 μL of DMEM medium in 96-well plates and grown for 24 h. The cells were then exposed to a series of HPEI nanogels, PEI2K, or PEI25K with different concentrations for 48 h, and the viability of cells was measured using the MTT method.

Western Blot Analysis. Western blot analysis was used to determine whether VSVMP was expressed by C-26 cells after transfection with pVSVMP/HPEI complexes.[51] Briefly, the lysates of C-26 cells transferred with pVSVMP/HPEI or pEP/HPEI complexes for 48 h were separated by SDS-PAGE. Gels were electroblotted with

Sartoblot onto a poly(vinylidene difluoride) (PVDF) membrane. Membrane blots were blocked at 4 °C in 5% nonfat dry milk, washed, and probed with rabbit sera at 1:500. Blots were then washed and incubated with a biotinylated secondary antibody (biotinylated mouse antirabbit IgG or IgM), followed by transfer to Vectastain ABC (Vector Laboratories). β-Actin (Sigma, USA) was employed as the internal standard.

Anticancer Activity of pVSVMP/HPEI Complexes on C-26 Cells *In Vitro*. The anticancer activity of the pVSVMP/HPEI complex on the C-26 cell proliferation was studied *in vitro*. C-26 colon carcinoma cells (2×10^5) were grown in 6-well plates and incubated for 24 h to 30% confluence. DNA (pVSVMP or pEP)/HPEI complexes (2 μg DNA/10 μg HPEI) were prepared in DMEM medium without serum, and normal saline (NS) was also used as a control agent. Cells were incubated with pVSVMP/HPEI complexes, pEP/HPEI complexes, or HPEI nanogels for 6 h, and the medium was then replaced by 2 mL of DMEM supplemented with FCS and incubated for an additional 48 h. The number of viable cells was determined by a trypan blue dye exclusion test, and the percentage of inhibition was calculated by the following formula as previously described: inhibition % $= [(N - N_T)/(N - N_0)] \times 100$, where N is the number of untreated cells cultured for 48 h, N_0 is the cell number on day 0, and N_T is the number of treated cells cultured for 48 h.[11]

Apoptosis assays were performed on pVSVMP/HPEI complexes treated C-26 cells. C-26 cells were treated with pVSVMP/HPEI complexes, pEP/HPEI complexes, HPEI nanogels, or NS for 48 h. Quantitative evaluation of cellular apoptosis was carried out by flow cytometric analysis using PI staining method.

pVSVMP/HPEI Complex Treats Mice Bearing C-26 Tumor *In Vitro*. For the abdominal cavity metastatic model, BALB/c mice (6−8 weeks old) were intraperitoneally injected with 0.2 mL of cell suspension containing 2×10^5 C-26 cells on day 0. On day 3, the mice were randomized into 4 groups (5 mice per group) and numbered. The four groups were intraperitoneally injected with 10 dosages of normal saline (control), HPEI nanogels (HPEI, 25 μg), pEP/HPEI complexes (EP/HPEI, 5 μg/25 μg), or pVSVMP/HPEI complexes (VSVMP/HPEI, 5 μg/25 μg) in the following 10 days. The weight of mice was recorded every day. On day 16, the mice in the control group were very weak; on that day, all mice were killed by cervical vertebra dislocation, and their tumors were immediately harvested, weighed, and analyzed. For the tumor growth study, T_w/C_w, the ratio of the mean tumor weight in the treated mice (T_w) divided by that of the control group (C_w), was determined. To further study the therapeutic effect of pVSVMP/HPEI complexes on C-26 colon carcinoma in this model, the survival time of those mice treated with the protocols described above was recorded (five mice in each group). Mice were sacrificed when they became moribund, and the day of sacrifice of the mouse was considered as its survival time.

For the pulmonary metastatic model, mice were intravenously injected with 0.2 mL of cell suspension containing 1×10^5 C-26 cells on day 0. On day 5, the mice were randomized into 4 groups (5 mice per group) and numbered. The four groups were intravenously injected with 10 dosages of normal saline (control), HPEI nanogels (HPEI, 25 μg), pEP/HPEI complexes (EP/HPEI, 5 μg/25 μg), or pVSVMP/HPEI complexes (VSVMP/HPEI, 5 μg/25 μg) in the following 10 days. The mice were weighed every day. On day 18, all mice were killed by cervical vertebra dislocation, and their lungs were immediately harvested, weighed, and analyzed. The metastatic nodules of C-26 colon carcinoma were numbered in each lung.

Histological Analysis. A commercially available TUNEL kit (Promega, Madison, WI) was used to analyze apoptotic cells within abdominal cavity metastases of C-26 carcinoma tissue. This analysis was performed following the manufacturer's protocol.

Statistical Analysis. Data were expressed as the means with 95% confidence intervals. Statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS software. Survival was assessed with the Kaplan−Meier method. For all tests, $P < 0.05$ was considered to be statistically significant.

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