Polyethyleneimine (PEI) has been extensively used as a common gene carrier due to its high gene transfection efficiency. PEI1.8k shows significantly lower cytotoxicity than its high molecular weight counterparts. However, it also has the problem of low gene transfection efficiency. To address the dilemma, a highly effective crosslinker (DTME) was synthesized to react with PEI1.8k to obtain CS-PEI1.8k. The reaction showed several advantages, such as a fast process in room temperature within nine hours with the product which can directly complex with DNA after removing the solvent. The ability of CS-PEI1.8k to agglomerate with DNA was proven by particle size, zeta potential, and gel retardation assays. The cytotoxicity and cell internalization capacity of CS-PEI1.8k were tested to verify the transfection capacity of CS-PEI1.8k. Moreover, we also studied the mechanism of the relatively high level of gene transfection by this binary complex compared with PEI25k.

1. Introduction

Gene therapy as a promising treatment has been widely used for the cure of various sicknesses of people [1, 2]. However, because of their rapid degradation in systemic circulation and low cell internalization efficiency, suitable DNA vectors are needed to promote their clinical application [3]. Although it has been used in clinical trials for many years, unnecessary immune response and abnormal gene expression prevent further clinical translation of viral vectors [4]. Nonviral vectors have attracted much attention because of their affordable mass production and the possibility of customizing structures and functions [5-7]. Cationic lipids and polymers make up many nonviral vectors [8]. In recent years, cationic polymers have attracted wide attention due to their limited immunogenicity [9], wonderful ability with regard to agglomerating DNA, and flexibility of structural modification. The nanoparticles formed by the electrostatic interaction between the cationic polymer and DNA keeps the DNA from decreasing. Poly-lysine (PLL) [10], polyamide amine (PAMAM) [11], chitosan [12], polyethylenimine (PEI), and polyphosphate as nonviral gene vectors have been extensively studied. Among them, branched PEI25k is one of the most widely used polymers. It is thought that its charge promotes cell endocytosis and passes the negatively charged cell surface, and PEI greatly induces the so-called “proton sponge effect,” enabling it to escape the internal trafficking pathway, leading to excellent transfection efficiency [13]. The drawback for PEI25k is its inherent cytotoxicity, which is caused by its positive charges that can compromise the membrane of cells and mitochondria, thus resulting in cell death. To solve the dilemma, all kinds of methods have been attempted. On the one hand, polyethylene glycol [14] or the macromolecules are grafted onto the cationic polymers to reduce the positive charges on its surface, which can greatly relieve the cytotoxicity. However, grafting hydrophilic fragments will also lead to reducing the transfection ratio. On the other hand, some studies bring in hydrophobic segments...
to PEI to lower its cytotoxicity and simultaneously keep its transfection rate, but the drawback of this method is that PEI25k is still not biodegradable.

The cytotoxicity of PEI1.8k was significantly lower than that of its high molecular weight, but its transfection efficiency also decreased. To solve this problem [15], a variety of modification strategies has been developed to improve the gene transfection efficiency of low molecular weight PEI [16–18]. For example, low molecular weight PEI was grafted onto polylactic acid [15], chitosan [19], or polycarbonates [20]. The transfection efficiency was greatly improved, and the cytotoxicity was much lower than that of PEI25k. The introduction of hydrophobic fragments into low molecular weight PEI is another effective strategy to improve the efficiency of gene transfection [21]. However, the gene transfection efficiency is reduced due to the close accumulation of genes, which has been found to prevent the release of therapeutic genes at the target site after internalization [22]. Therefore, the effective transmission and release of genes into the target site should be the two decisive factors for good gene transfection efficiency. Intracellular pH and redox potential are two common cellular reactivity studies [23]. Although pH stimulates the polymer to degrade in the acidic environment of the endosome or lysosome, resulting in the release of therapeutic genes, therapeutic genes released in the endosome or lysosome generally reduce bioavailability at target sites such as the cytoplasm and nucleus [24]. In contrast, redox potentials can trigger the degradation of redox potential-responsive polymers after escape from the internal lysosomal chamber and then release therapeutic genes in the cytoplasm rather than in internal lysosomes, resulting in better bioavailability than pH-induced reactions [25].

In this study, binary complexes of plasmid DNA and CS-PEI1.8k were prepared. Firstly, we synthesized a highly effective crosslinker DTME [26]. Subsequently, DTME was reacted with PEI1.8k, thus obtaining the product named CS-PEI1.8k. The new crosslinker showed several advantages compared with other crosslinkers, such as the reaction could proceed at room temperature within only nine hours and the prepared product was water-soluble, which could directly complex with DNA. CS-PEI1.8k could effectively stabilize and protect the payload before reaching the target location. The cytoplasm escapes from the internal lysosomal chamber and reaches the target cell (Scheme 1). It was expected that DNA could be released from the complex triggered by the redox potential of the cytoplasm. The ability of CS-PEI1.8k to agglomerate pDNA was proven by grain diameter and gel retention experiments. The in vitro transfection and cell internalization capacity of CS-PEI1.8k were tested to verify the transfection capacity of CS-PEI1.8k. We also studied the mechanism of a relatively high level of gene transfection efficiency compared with PEI25k.

2. Results and Discussion

2.1. Synthesis and Characterization of CS-PEI. In this work, CS-PEI1.8k was prepared by the crosslinking of PEI1.8k with DTME (Scheme 2). The crosslinking process reacted quickly and took only nine hours. After that, the purification was simple without dialysis and lyophilization. The product could be used for transfection after removing the solvent. ^1^H NMR spectra, FTIR spectra, and Gel permeation chromatography (GPC) analysis were used to characterize the structure of the products. As shown in Fig. S1–S2, the crosslinking agent DTME was successfully synthesized from cystamine. The ^1^H NMR spectra of CS-PEI1.8k crosslinked by DTME are shown in Fig. S3 with the peak at 2.0–3.0 ppm representing the PEI1.8k segment. The FTIR result of CS-PEI1.8k indicated successful combination of PEI1.8k and DTME (Fig. S4). The molecular weight result of CS-PEI1.8k was 6.52 × 10^3^, which could demonstrate the successful combination of PEI1.8k (the molecular weight result was 1.01 × 10^3^) and DTME (Fig. S5), and the PDI results demonstrated homogeneous molecular weight distribution. These results indicated the successful preparation of CS-PEI1.8k. To explain that the reducing agent DTT can break the disulfide bonds, the GPC analysis was conducted again. From the experimental results can be seen that the disulfide bonds were broken by DTT. The molecular weight was reduced from 6.52 × 10^3^ to 1.11 × 10^3^ (Fig. S5). This change was more conducive to the release of DNA, which improved the efficiency of gene transfection.

2.2. Characterization of CS-PEI/pDNA Complex. The ζ-potential and particle size of the complex could affect its infiltration in tumor tissues. As shown in Figure 1(a), the particle size was gradually compressed with its increased content of CS-PEI1.8k. When the particle size of the complex increased approximately 200 nm, the particle infiltration in tumor tissues would enhance with an improved endocytosis. As shown in Figure 1(b), with the increase of the composite ratio, the surface ζ-potential of the complex gradually improved. The transmission electron microscopy in Figure 2 shows that the CS-PEI1.8k has the ability to condense DNA into spherical and compact nanoparticles, similar to PEI1.8k. The size is about 100–200 nm, which is consistent with the result of DLS measurement. Moreover, DNA can be released when the crosslinked PEI is degraded by DTT. Therefore, just the right surface charge and particle size will be beneficial to improve the stability and long cycle performance of the system.

The DNA combination ability was evaluated by using the gel retardation assay. A key step for gene expression was that the DNA was released from the complex. As shown in Figure 3, their electrophoretic mobility on the agarose gel was used to detect the composition of the complexes under different N/P ratios with and without DTT. Only the DNA in the CS-PEI1.8k group was more easily displaced from the complex after DTT addition. The toxicity of the gene carriers to HeLa and B16F10 cells was detected by (MTT) colorimetric assay. As shown in Figure 4, in comparison with PEI1.8k and its crosslinked products, PEI25k had very high cytotoxicity. The toxicity of CS-PEI1.8k was in proportion to its concentrations in HeLa and B16F10 cells. As the crosslinked CS-PEI1.8k increased in molecular quantity, the cytotoxicity will also increase. However, there is no significant difference between
CS-PEI1.8k and PEI1.8k, which can be seen from Figure 4. The results indicated that the molecular weight of PEI was an important factor for its cytotoxicity.

2.3. Transfection Activities. The transfection efficiency of PEI1.8k and its crosslinked products was estimated by luciferase assay in HeLa and B16F10 cells. PEI25k was used as the control. Figures 5(a) and 5(b) show the transfection efficiency of the different transfection groups in HeLa and B16F10 cells at 48 hours after transfection, respectively. It could be seen that the crosslinked products have good transfection properties in both types of cells. The molar ratio of PEI1.8k and DTME was 1/0.8 (1-0.8 in Figure 5) which showed the best graft ratio in both cells. The optimal transfection ratio was 20/1, which significantly improved transfection efficiency.
To investigate the different cell internalization efficiency between PEI25k, PEI1.8k, and CS-PEI1.8k, cell uptake of fluorescent-labeled DNA (Cy5-DNA) was monitored by flow cytometry in HeLa cells. As shown in Fig. S6 and Figure 6(a), CS-PEI1.8k could effectively mediate endocytosis and achieve the best level of internalization efficiency. The weight rates of PEI1.8k and Cy5-DNA were 10/1 (1-10 in Figure 6(a)). The CS-PEI1.8k presented the improved transfection efficiency, compared with the results of PEI25k and PEI1.8k (Figure 6(b) and Fig. S7).

2.4. Model Analysis of the Improved Transfection Ratios. The intracellular trafficking of three-gene carriers was observed by CLSM. As shown in Figure 7, the results demonstrated that only the weak signal of PEI1.8k/Cy5-DNA was observed in cells after 4 h incubation, while the CS-PEI1.8k/Cy5-DNA group had higher endocytosis efficiency, and the PEI25k group had more fluorescence signals than PEI1.8k and CS-PEI1.8k. There was a very clear association between transfection and endocytosis. From microscopic observation, PEI25k with a more positive charge and higher molecular weight caused higher endocytosis ability. The relatively good transfection effect of CS-PEI1.8k was due to the redox responsive-based gene release.

3. Discussion

Gene therapy is an emerging medical method, which is expected to fundamentally cure congenital and acquired diseases. However, the preparation of low toxicity, high efficiency, and stable gene vectors is a key factor limiting its development. Polyethylenimine is one of the most studied and effective nonviral gene vector materials, especially the 25k hyperbranched polyethylenimine (PEI25k), which has become the “gold standard” to measure the performance of nonviral gene vectors. Polyethylenimine is a kind of synthetic polycation. It is easy to synthesize and has been widely used as a floculating agent. The molecular structure of PEI
can be divided into branched and linear forms. Branched PEI is directly produced by the ring-opening polymerization of aziridine under the catalysis of acid. The industrial production is very simple, but it is not recommended to synthesize aziridine in the laboratory, because it is a flammable, explosive, and highly toxic gas at room temperature.

It was found that the toxicity of polyethylenimine could be reduced by decreasing the relative molecular weight. The transfection efficiency will decrease at the same time. Because the surface of PEI25k has a high density of positive charge, the cytotoxicity is greater, and it aggregates easily under physiological conditions and is hard to be degraded. The cytotoxicity of low molecular weight PEI is lower and modification can improve the transfection efficiency. PEI1.8k shows significantly lower cytotoxicity than its high molecular weight counterparts. However, it also has the problem of low gene transfection efficiency. To address the dilemma, a highly effective crosslinker (DTME) was synthesized to react with PEI1.8k to obtain CS-PEI1.8k.

The data in this study first revealed the synthesis method of the material. The crosslinking process reacted quickly and took only nine hours. The elimination of dialysis and lyophilization operations makes the purification process simpler, and transfection tests can proceed after solvent removal. This is a flawless synthesis method, which not only saves a lot of time but also solves the waste of raw materials in the process of preparing the material. It is so important to accelerate the synthesis speed and improve the efficiency.

The conclusions can be drawn from the 1H NMR spectra results and PDI results; the PEI1.8k and DTME have been combined successfully. The appropriate particle size and surface potential result in efficient endocytosis of the complex by tumor cells. When the particle size of complex particles in an ideal transfection ratio range was about 200 nm, the infiltration effect and the endocytosis of particles in the tumor tissue both were improved. Transmission electron microscopy analysis can be used as a supplementary evidence to jointly verify the morphology of the composite particles, which can be clearly seen from the experimental results; the CS-PEI1.8k was similar to PEI1.8k with the size about 100–200 nm. A key step for gene expression was that the DNA released from the complex. The ability of DNA released was investigated by gel retardation electrophoresis assay under different N/P ratios with and without DTT.
Compared with the PEI25k/DNA and PEI1.8k/DNA, it can be seen that only the DNA in the CS-PEI1.8k group more easily escaped from the complex after DTT addition. Transfection efficiency is the best index to evaluate the quality of the vector. The main task here was to show the transfection efficiency of the different transfection groups in HeLa and B16F10 cells at 48 hours after transfection, respectively. This indicates that the CS-PEI1.8k has good transfection properties in both types of cells, especially in the crosslinking ratio was 1/0.8. Both FCM and CLSM analyses were used to evaluate the endocytosis of endocytosis efficiency. High endocytosis means more effective genes entering the cells, and more genes were delivered to the tumor. The best uptake effect of CS-PEI1.8k was due to redox responsive-based gene release. These findings provide guidelines for the design of gene delivery vectors, and the relationship between physicochemical characters, systemic toxicity, transfection efficiency, and endocytosis efficiency was systematically studied. It is still very much needed.

4. Conclusion

In conclusion, CS-PEI1.8k was obtained by a highly effective crosslinker DTME reacting with PEI1.8k. The results of particle size, zeta potential, and gel retardation experiments proved that the CS-PEI1.8k combination with DNA became spherical nanoparticles. Compared with PEI25k or PEI1.8k, the CS-PEI1.8k exhibited lower cytotoxicity and excellent transfection efficiency. Cell internalization was evaluated by investigation of the transfection capacity about CS-PEI1.8k. The results of FCM and CLSM further demonstrated that the redox-sensitive gene vector of CS-PEI1.8k had good gene delivery ability.

5. Experimental Section

5.1. Materials. Triethylamine (99%), maleic anhydride (99%), cysteamine hydrochloride (98%), and DTT (>98%) were purchased from Energy Chemical (Shanghai, China). Hyperbranched PEI (Mn = 1.8 kDa and Mw = 25 kDa) was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA. The calf thymus DNA in the experiment was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from a big island of USA. The BCA protein test kit in our experiment was purchased from Pierce in Rockford, Illinois, USA.
5.2. Preparation of Cystamine Free Base. In the experiment, we carefully dissolved and diluted 30 g of cysteine hydrochloride (0.14 mol) in distilled water (50 mL); afterwards, we gradually added chloroform (150 mL). We used ice baths (ice and NaCl) to cool the mixture to -5 °C. In the experiment, we used a 40% sodium hydroxide aqueous solution on ice (in which we set the solute as 40% sodium hydroxide), which was slowly dropped into the mixture at a temperature not exceeding 0 °C. It could be observed that under continuous stirring, after stirring 15 min at the same temperature, we found that the organic layer was completely separated, and the remaining residue was treated with an additional 100 mL chloroform as planned. Then, the bound organic layer was evaporated in a magnesium sulfate bed at nearly room temperature of 30 °C to dry the sample and use it as soon as possible after preparation. The product was characterized by 1H NMR (CDCl3) as shown in Fig. S1.

5.3. Synthesis of Crosslinker Dithiobis(maleimido)ethane (DTME). Cystamine (4 g, 26.26 mmol) was soluble in 100 mL acetone; afterwards, maleic anhydride (5.15 g, 52.53 mmol) was aminated to the mixture. The sudden precipitation of dicarboxylic acid was observed, and the mixture was stirred for one hour to complete the reaction. Triethylamine (2 mL, 27.2 mmol) and sodium acetate (0.03 g) were added to the reaction mixture. The mixture was then slowly heated and refluxed, and acetic anhydride (8 mL, 72.5 mmol) was added at the same time. The mixture was allowed to reflux for 3 hours, and then, the acetone was evaporated. The residue was dissolved in dichloromethane (100 mL) and washed with saturated NaHCO3 solution (50 mL) to remove the triethylamine salt. The dichloromethane phase was evaporated after drying with magnesium sulfate. The residual acetic anhydride was removed by cyclohexane azeotropic distillation. The crude compound was then purified by column chromatography (40% ethyl acetate/hexane). The product was characterized by 1H NMR (CDCl3) as shown in Fig. S2.

5.4. Synthesis of Redox-Responsive Polymers via Maleimide-Conjugate Addition. PEI1.8k (2 g, 1.11 mmol) and DTME (0.5 g) were dissolved in methanol, which was stirred for 9 hours. Afterwards, the solvent was removed in vacuum to obtain CS-PEI1.8k. We adjusted the addition molar ratio of PEI1.8k and DTME. They are 1/0.7, 1/0.8, and 1/0.9. The product was characterized by 1H NMR (CDCl3) as shown in Fig. S3.

5.5. Characterization of CS-PEI1.8k. The 1H NMR spectra was carried out on the BrukerAV-400 spectrometer (Bruker, Ettlingen, Germany). The measurements were carried out at room temperature with CDCl3. The Mn, Mw, and polydispersity index (PDI) of CS-PEI1.8k were measured by Waters 515 GPC system. The system was equipped with a linear 7.8 × 300 mm column (Waters, Milford, MA, USA), an 18-angle laser scatterometer (Wyatt Technologies, USA), and an Optilab DSP interference refractometer (Wyatt Technologies, USA). NaAc/HAc buffer (0.35 M) was used as the eluent with the pH 4.4. The flow rate was 0.5 mL/min. The calibration curve was generated by a monodisperse PEG standard with a molecular weight range of 1.8-400 kg/mol (purchased from Waters Company).

5.6. Polymer/Plasmid DNA Complex Characterization. Zeta potential and particle size of the complex was determined by particle analyzing instruments (Zeta-PALS, Brookhaven, NY). After incubating 20 min at room temperature, different mass ratio carriers/DNA (calf thymus DNA) ranging from 1 to 40 were measured. The ability of the complex to bind to plasmid DNA was evaluated by agarose gel electrophoresis. The PEI25k and CS-PEI1.8k solutions with a weight ratio of 0 to 0.6 were added to 0.5 μg of pDNA, and the total volume of the complex was determined to be 15 μL. Before gel electrophoresis, the complex was incubated for 30 min at room temperature. To further verify, dithiothreitol (DTT) was continuously added to the complexes of different concentrations under the same experimental conditions. 1% agarose gel electrophoresis was performed according to the above steps.

5.7. Culture Cell. HeLa cells were cultured in high glucose DMEM medium with 10% FBS, which has been heat inactivated; 100 units per milliliter of penicillin, and 100 μg per milliliter streptomycin and cultured in a 5% CO2 incubator at 95% humidity at 37 °C. The same culture conditions were used in B16F10 cells except for the 1640 medium. The stable transfection of HeLa and B16F10 cells were provided by Northeast Normal University.

5.8. Cytotoxicity Assay. The biocompatibility of CS-PEI1.8k was determined by the MTT method. HeLa cells were seeded in 96-well plates, and 1.0 × 104 cells in each well were cultured for 24 hours, and then, the culture medium was taken out and replaced with 180 μL of fresh medium. CS-PEI1.8k was added to each well with gradient concentrations. The culture plate was put back to the culture apparatus for 48 hours. After the experiment, 20 μL MTT solutions were added to each well with concentrations of 5 mg/mL in phosphate-buffered saline (PBS). The culture plate was sent back to the incubator. After 4 hours, the MTT solution was removed from each well carefully, and 200 μL dimethyl sulfoxide (DMSO) was added to dissolve the MTT methanide crystal. The culture plate was incubated again with 8 min before the absorbance was measured by a microboard reader (Bio-Rad) with enzyme-linked immunosorbent assay (ELISA). The cell viability (%) was computed according to the following equation: cell viability (%) = (Ab sample/Ab control) × 100, in which Ab sample represented the absorbance of CS-PEI1.8k-treated cells and the Ab control represented the absorbance of untreated cells. The MTT experiment was repeated at least three times.

5.9. In Vitro Transfection. HeLa and B16F10 cells were prepared in 96-well plates, and 1.0 × 104 cells in each well contained 200 μL 10% serum in the culture medium 24 hours before transfection. Before starting, the old culture medium was replaced with 10% fresh medium, the polymer/PGL3 (0.2 μg pDNA) complex with gradient ratio was added to
each well, and the cells were incubated at 37°C for 48 hours later. The sterile culture medium was thrown away, and the cells were washed with PBS for four times. After the cells were thoroughly lysed with the cell lystate buffer at 50 μL per well (Promega), the luciferase gene expression was counted by luciferase reporter gene detection kit and photometer (GloMax20/20, Promega). 50 μL luciferin was added to the 20 μL cell lystate per well. To detect the protein content, the BCA protein assay kit (Pierce, Rockford, IL, USA) was used. The in vitro transfection experiment was repeated at least three times.

5.10. Cellular Uptake Assay. For flow cytometry (FCM) analysis, Cy5-labeled pGL3 plasmid DNA (Cy5-DNA) and copolymer were used to form a complex to study cell uptake in vitro. HeLa cells (2.0 × 10^5 cells per well) were seeded in a 6-well plate containing 10% serum medium 2 mL for 24 hours, and the original medium was replaced by 2.0 mL of fresh growth medium containing the pGL3 complex. The cells were incubated at 37°C for 4 hours; the collected cells were washed with PBS, separated with trypsin containing 0.25% in PBS, and then blended with 500 μL PBS. Finally, the internalization efficiency of the polymers was measured by flow cytometry (FACScalibur, Becton-Dickinson, San Jose, CA, USA). The uptake behavior was observed by confocal laser scanning microscopic (CLSM); we labeled the plasmid DNA with Cy5 and labeled the polymer with fluorescein isothiocyanate (FITC). HeLa cells were cultured in a 6-well plate with an initial density of 2.0 × 10^5 cells per well. Before fixing the cells with 4% paraformaldehyde, the cells were affected with the complex at 37°C for 4 hours. Fixation completed, the cover slides were washed for three times with PBS and then was stained with 1 μL 4,6-diamino-2-phenylindole (DAPI, 1 mg·mL⁻¹) for 10 min. The cover was cleaned with PBS many times, sealed with glycerol, and then visualized with CLSM (Leica TCSSP2, Leica Microsystem, Wetzlar, Germany).

5.11. Statistical Analysis. The two-tailed Student’s t-test was used for statistical analysis. All data are expressed as the average standard deviation of independent measurements. When the p value is less than 0.05, it is statistically significant. Statistical significance was set at *p < 0.05, **p < 0.01, and ***p < 0.001 which were deemed extremely significant.

Data Availability

The data used to support the findings of this study are included within the article. Any additional data that support the findings of this study are available from the corresponding authors upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Materials

The supplemental files include Figure S1 to Figure S7. The structural properties are reported in Figure S1 to Figure S5, and the detailed cell internalization results are described in Figure S6 and Figure S7. (Supplementary Materials).

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