Synthesis and Characterization of Quaternized Poly(β-amino ester) for Highly Efficient Delivery of Small Interfering RNA

Yun Liu, † Jing Chen, † Yue Tang, †* Shuhan Li, Yushun Dou, and Jiiewen Zheng

Department of Pharmacy, China Pharmaceutical University, Nanjing 211198, PR China

ABSTRACT: The development of non-viral vectors for gene delivery has gained attention over the past decades. Specifically, poly(β-amino ester) (PBAE) has shown great potential for improving the delivery of gene therapeutics. It has been observed that low-molecular-weight PBAE displayed low transfection activities, while quaternization could enhance the transgene expression efficacy of PBAE. Herein, PBAE quaternary ammonium salt (PBAEQAS) was synthesized to increase the positive charge of the polymers, which resulted in an increase in siRNA binding efficiency based on self-assembly electrostatic interaction. Specifically, the nanoparticle surface was positively charged, which increased the uptake ability of siRNA. Compared with acrylate-PBAEQAS/siNC nanoparticles and amine-PBAEQAS/siNC nanoparticles, acrylate-PBAEQAS/siSurvivin nanoparticles and amine-PBAEQAS/siSurvivin nanoparticles induced more-efficient cell apoptosis and gene silencing. All these results suggest that PBAEQAS would be a promising gene delivery vector for cancer treatment.

KEYWORDS: poly(β-amino ester), quaternization, nanoparticles, siRNA, transfection

1. INTRODUCTION

Gene therapy is an important part of cancer treatment, and it can be achieved by substitution of altered genes, inhibition of oncogenes, or insertion of new genes. 1 A successful gene delivery system promises efficient gene transfection. The carriers for gene delivery are divided into viral vectors and non-viral vectors. Viral vectors pose high gene transfer efficiency in vivo, but they are more likely to cause immunogenicity because of their strong innate immune response. 2 and the viral vectors also have virus-associated toxic side effects. 3 Meanwhile, non-viral vectors have the advantages of reduced cytotoxicity, targeting properties, and high transfection efficiency. 4-6 Non-viral vectors usually refer to liposomes, cationic polymers, peptides, and inorganic materials. 7-9

In the past decades, a number of polymeric materials have been utilized in formulating gene delivery systems, such as poly(lactic-co-glycolic acid) (PLGA), chitosan, poly(amidoamine) (PAMAM), and poly(l-lysine) (PLL). 10-12 Poly(β-amino esters) (PBAE) are promising materials due to their biodegradable and biocompatible nature. Its special chemical structure endows PBAE with availability for further modification. 5,13,14 To date, a library of PBAEs have been synthesized. Among them, some types of PBAEs have higher in vitro gene transfection efficiencies than poly(ethyleneimine) (PEI); however, there still exist other PBAEs having low transfection ability before modification. Thus, this polymeric material is complicated and requiring further study. 15-17 It has been observed that PBAEs with high molecular weight were able to mediate effective intracellular gene delivery, but they also showed considerable toxicities. 18 While low-molecular-weight PBAE posed low transfection efficiency, the low positive charge density may reduce its ability to compact small interfering RNA (siRNA) in physiological conditions. 19

When the molecular weight of PBAE was less than 10 kDa, the PBAE could not effectively condense siRNA 20,21 but lower molecular weight PBAE reacted more quickly than higher molecular weight PBAE in terms of being able to bind to siRNA. 22 It had also been found that for PBAE/siRNA nanoparticles that are generally prepared in acidic buffer solution, the particle size increased significantly when the nanoparticles were transferred into physiological conditions (pH 7.4). 23

Recently, most studies have focused on various derivatives of polymers to improve the efficiency of gene delivery. For example, it was found that grafting poly(ethylene glycol) (PEG) in PBAE could enhance the stability of PBAE/DNA complexes. 6,24 Highly branched PBAE has been deeply studied, and it was proved that branched PBAE could significantly improve transfection efficiency. 25-28 Low-molecular-weight PEI-terminated PBAE could improve the gene delivery efficiency over that of the polymer before modification. 13 Moreover, structural modifications, such as changing the quantity and types of amines, have been implemented to enhance siRNA delivery efficiency. 29,30 Miryala et al. proved that quaternization of aminoglycoside-derived polymers could enhance the gene delivery efficiency. 31 By quaternary ammonium (QA) reaction, the positive charge density of
The objective of this study was to explore a novel kind of vector based on PBAE with enhanced siRNA delivery capability by increasing the positive charge density through quaternization. Scheme 1 shows the synthesis of PBAE quaternary ammonium salt (PBAEQAS), the preparation of nanoparticles, and the delivery of nanoparticles for cell transfection. First, PBAE was synthesized by a typical Michael addition reaction, and QA reaction was applied to prepare PBAEQAS. Second, the siRNA retardation assay was performed and the stability of the formulations evaluated. Third, the impact of intracellular delivery of such polymers on the cellular uptake and mechanisms, apoptosis, and gene silencing of A549 was assessed. We then developed these
vectors to deliver survivin siRNA (siSurvivin) to inhibit the expression of survivin genes in cancer cells. The influence of amine end-modified PBAEQAS and acrylate end-modified PBAEQAS on transfection efficiency was investigated as well.

2. MATERIALS AND METHODS

2.1. Materials and Cell Culture. 1,4-Butanediol diacylate, 4-amino-1-butanol, and thiazolyl blue tetrazolium bromide (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) were purchased from D&B Biological Science and Technology Co. Ltd. (Shanghai, China). Agarose was obtained from Solarbio Biotech Co. Ltd. (Beijing, China). JI Red was purchased from Biolite Biotech Co. Ltd. (Tianjing, China). Ribonuclease A (RNase A), diethyl pyrocarbonate (DEPC), chlorpromazine, Genestin, and Amiloride were purchased from Aldrich Chemical Co. (Shanghai, China). Filipin III was obtained from Cayman Chemical Co. (Ann Arbor, MI). Fetal bovine serum (FBS, Sijiqing) was purchased from Zhejiang Tianhang Biotechnology Co. Ltd. (Hangzhou, China). Trypsin, penicillin, streptomycin, RPMI 1640 cell culture medium, Hoechst 33342, and Trizol total RNA extraction reagent were obtained from KeyGen BioTech (Nanjing, China). LysoTracker Red was purchased from the Beyotime Institute of Biotechnology (Haimen, China). Annexin V-FITC and propidium iodide (PI) apoptosis assay kit were purchased from Xinxie Biotech Co. Ltd. (Shanghai, China). The quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) quantitation kit was purchased from GenePharm (Shanghai, China). The siRNA targeting survivin mRNA (siSurvivin), FAM-labeled siRNA, and negative control siRNA (siNC) were synthesized by GenePharm (Shanghai, China). The sequences of siRNA are as follows: siSurvivin, sense: 5′-GGACCCACCGCUACU-3′, antisense: 5′-UGUAGAGAUGCGUGU-3′; siNC, sense: 5′-UUCUCCGAACGUGU-3′, antisense: 5′-ACGUGACUCCGU-3′. All the reagents and chemicals were of analytical grade.

A549 cells (human lung adenocarcinoma cells), obtained from American Type Culture Collection (ATCC), were cultured at 37 °C in a 5% CO₂ incubator supplemented with complete RPMI 1640 medium (containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin) and allowed to attach overnight for subsequent experimentation.

2.2. Synthesis of PBAE and PBAE Quaternary Ammonium Salt (PBAEQAS). PBAE was synthesized following a two-step procedure described previously.13,32,33 Briefly, 1,4-butandiol diacrylate and 4-amino-1-butanol (at 1:1 molar ratio of amine:diacrylate) were added into a round-bottom flask, and the reaction solution was stirred at 90 °C for 24 h under the protection of nitrogen. To obtain amine end-capped modified PBAE, additional amine monomers were added, and the final solution was stirred for another 1 h at room temperature. Tertiary amine groups of PBAE were converted to QA by reaction with iodomethane (Scheme 2).34 A certain amount of PBAE solution was dissolved in 10 mL of isopropyl alcohol, and then iodomethane (at 2:1 molar ratio of iodomethane/PBAE) was added dropwise. The reaction mixture was stirred at 60 °C for 16 h. The solution was then removed, and the retained solid was washed three times with ethyl alcohol, followed by lyophilization for 24 h. The structures of the synthesized polymers were confirmed by 1H NMR and FT-IR analysis. The 1H NMR spectra were obtained on a Bruker AVANCE 500 MHz spectrometer. All measurements were performed at 300 K, using pulse accumulation of 64 scans and the LB parameter of 0.30 Hz. D₂O was used as the solvent for dissolving polymers. The average molecular weight of the polymers was determined by gel permeation chromatography (GPC, Shimadzu, Japan) using an instrument equipped with a refractive index detector (RID-10A, Shimadzu, Tokyo, Japan). The SEC-300 column (300 × 7.8 mm, 5 μm) was eluted with doubly distilled H₂O at a flow rate of 1 mL/min 40 °C. Samples of PEG with average molecular weights in the range of 3070–21600 Da were used as standards for calibration.

2.3. Preparation and Characterization of Nanoparticles. The polyplexes were formulated by mixing PBAEQAS and siRNA at different weight ratios. RNase-free DEPC-treated ultrapure water was used as solvent for all solutions of siRNA. Briefly, the PBAEQAS stock solutions in DMSO (100 mg/mL) were diluted with ultrapure water to 1 mg/mL. This solution was then mixed with a solution of siRNA (100 ng/μL), vortexed for 10 s, and incubated at room temperature for 30 min. Analysis of particle size distribution and zeta potential was performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom). To assess siRNA retardation, the resulting nanoparticles were characterized by agarose gel electrophoresis. The nanoparticles were added to wells of agarose gel (1%, containing 0.01% JI Red). The samples were run at 80 V for 30 min, visualized by UV illumination. In order to investigate the stability of the formulation, the nanoparticles (each group contained 50 nM siRNA) were incubated in the presence of 25 mg/mL RNase A at 37 °C.35 After 20 min, the samples were heated in a water bath at 80 °C to stop the reaction, and then the resulting solutions were treated with heparin and investigated by agarose gel electrophoresis.

2.4. Cytotoxicity Assay. The cytotoxicity of A549 cells were measured via the MTT assay. A549 cells were seeded on 96-well plates at a density of 2 × 10⁴ cells/mL and then cultured in complete medium RPMI 1640 for 24 h. The medium was replaced with serum-free RPMI 1640 (200 μL) containing various levels of nanoparticles. After incubation at 37 °C for 24 h, the medium was exchanged with MTT solution (180 μL of serum-free RPMI 1640 and 20 μL of MTT (1 mg/mL)) and further incubated at 37 °C for 4 h. Finally, the medium was removed, 200 μL of DMSO was added, and the mixture was then incubated for 10 min. Absorbance was measured by using a Microplate Reader (Multiskan FC, USA) at 490 nm. Cell viability was expressed as a relative percentage compared with untreated cells.

2.5. Cellular Uptake and Mechanisms. A549 cells were seeded on 6-well plates at a density of 2 × 10⁵ cells/mL and incubated for 12 h. The he cells were then washed with PBS and incubated with serum-free RPMI 1640 containing nanoparticles for 2, 4, 6, and 24 h. Nanoparticles were prepared as described previously. Briefly, FAM-labeled siRNA (50 nM) was mixed with PBAEQAS (1 mg/mL) at a weight ratio of 50:1, vortexed for 10 s, and incubated at room temperature for 30 min in the dark. The cells were then washed with PBS three times and analyzed by flow cytometry (MACSQuant Analyzer 10, Miltenyi, Germany). Furthermore, fluorescence microscopy (Olympus IX53, Japan) was used to qualitatively observe the cells’ uptake. A549 cells were transfected with the nanoparticles for 6 h, fixed with 4% paraformaldehyde, and stained with Hoechst 33342.
Mechanistic studies of FAM-labeled siRNA-loaded nanoparticles on cellular uptake were performed at 4 °C for 30 min or after incubation with various endocytic inhibitors (sodium azide (1 mg/mL), chlorpromazine (10 mg/mL), hypertonic sucrose (450 mM), genistein (200 μM), filipin (5 μg/mL), and amiloride (13.3 μg/mL)) within a period of 30 min at 37 °C prior to the addition of polyplexes, and then the cells were incubated with the nanoparticles for 4 h at 37 °C. Cells incubated with nanoparticles in the absence of endocytic inhibitors at 37 °C for 4 h were used as control.36,37

2.6. Apoptosis of A549 Cells. A549 cells were seeded on 6-well plates and cultured overnight. The medium was then replaced with serum-free RPMI 1640. Nanoparticles were added to each test well. After 6 h of incubation at 37 °C, the medium was replaced with fresh medium containing 10% FBS, and the cells were cultured for another 42 h. The cells were washed twice with PBS and harvest by EDTA-free trypsin, re-suspended with 300 μL of binding buffer and 5 μL of annexin V-FITC, and incubated at room temperature for 15 min. Next, 5 μL of PI was added before the cells were analyzed by flow cytometry. All the operations were carried out under protection from light. The cells incubated with PBAEQAS/siNC nanoparticles and the cells incubated with naked siSurvivin were set as controls.

2.7. In Vitro Gene Silencing. A549 cells were seeded in 6-well plates with 2 mL of RPMI 1640 medium containing 10% FBS. After the cells showed 70–80% confluence, the culture medium was changed into fresh complete medium containing mocks, siNC-loaded nanoparticles, and siSurvivin-loaded nanoparticles, respectively. After 6 h incubation, the medium contained samples was replaced with fresh serum-free medium, and the cells were further cultured for another 42 h. The cells were then washed with PBS and lysed by Trizol reagent (KeyGEN BioTECH). The isolated total RNA was quantified by using a Nano 100 micro UV spectrophotometer (All for Life Science, China) at 260 nm. qRT-PCR was performed by using the SYBR Green I dye method according to the manufacturer’s instructions. A QuantStudio 3 real-time PCR system was used, and the qRT-PCR data were analyzed using the comparative CT(2−ΔΔCT) method.38

2.8. Statistical Analysis. All experiments were carried out in triplicate, and data are reported as the means ± SD. The Student’s t test was performed to determine the statistical significance of the difference group means at p < 0.005, 0.005 < p < 0.01, and P < 0.05.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of PBAE and PBAEQAS. The PBAEs were synthesized following a two-step procedure. First, acrylate-terminated PBAE (acrylate-PBAE) was obtained via the Michael reaction as previously described, and then additional 4-amino-1-butanol was added to obtain amine-PBAE (shown in Scheme 2A). In order to enhance the positive charge density, the tertiary amine groups in the PBAE were reacted with iodomethane, and the reaction was carried out in isopropyl alcohol (shown in Scheme 2B) to produce PBAEQAS. The chemical structures of PBAEQAS were characterized by 1H NMR and FT-IR spectra. A very strong 1H NMR peak at 3.2 ppm was observed in amine-PBAEQAS and acrylate-PBAEQAS compared to the parent polymer, indicating the presence of methyl groups of QA side chains.39 The methylene protons from amine monomer were present at
δ = 1.25–1.45 ppm, and the methylene protons from acrylate monomer were present at δ = 1.50–1.60 ppm. The signals at δ = 2.30–2.70 and δ = 3.30 ppm corresponded to the methylene protons close to the nitrogen atoms and methylene protons close to the oxygen atoms, respectively. The presence of methylene protons close to the carbonyl of the ester bonds was confirmed by the signal at δ = 3.60–3.90 ppm. The typical peaks of double bonds of acrylate at δ = 5.0–7.0 ppm demonstrated that the PBAE was end-capped with acryl groups (Figure 1C). As for amine-end-capped PBAE, there was no occurrence of this type of peak (Figure 1A). As shown in Figure 1B,D, the broad band at 3600–3200 cm⁻¹ is attributed to O–H. The band at 1731 cm⁻¹ is assigned to the C=O stretch of ester groups. After quaternization, a new characteristic peak located at 1560 cm⁻¹, assignable to methyl groups of the ammonium moiety, is seen in the FT-IR spectra. GPC

Figure 2. (A–C) Gel electrophoretic retardation analysis of polymer/siRNA nanoparticles at various weight ratios: (A) amine-PBAE and amine-PBAEQAS; (B) acrylate-PBAE and acrylate-PBAEQAS; and (C) RNase stability analysis of polymer/siRNA nanoparticles. (D) Size and zeta potential of PBAEQAS/siRNA nanoparticles.

Figure 3. (A) Cytotoxicity of various siNC-loaded nanoparticles at various concentrations (n = 6). (B) Internalization identification of nanoparticles in A549 cells as determined by flow cytometric analysis. (C) Quantification of cell internalization at various times, shown by mean fluorescence intensity (MFI; *P < 0.1, **P < 0.01). (D) Effects of endocytosis inhibitors and temperature on the internalization of siNC-loaded nanoparticles (n = 3). Relative uptake level (%) = MFI (treated group)/MFI (control group) × 100%.
traces of polymers are shown in Figure S1. The molecular weights of the polymers detected by GPC were $\sim 2.3$ kDa (amine-PBAE), $\sim 3.4$ kDa (acrylate-PBAE), $\sim 2.7$ kDa (amine-PBAEQAS), and $\sim 4.1$ kDa (acrylate-PBAEQAS).

3.2. Formation and Characterization of Nanoparticles. The formation of nanoparticles with appropriate size and positively charged surface is an important prerequisite for polyplexes to be internalized into cells. The naked siRNA is negatively charged. Cationic polymers can bind siRNA based on self-assembly electrostatic interactions in ultrapure water. The delivery efficiency of nanoparticles based on PBAE is determined by the size, surface charge, and other physicochemical nature of the material.41 The PBAEs with lower molecular weight had lower charge density. Furthermore, it had been proved that, when the weight ratio was lower than 100:1, PBAE could not effectively combine with siRNA in the retardation assay (Figure 2A,B). The size distribution and zeta potential of the nanoparticles were investigated by dynamic light scattering (DLS). When the weight ratio was 150:1, the size and zeta potential of the PBAE/siRNA were $275.6 \pm 11.04$ nm and $281.8 \pm 8.08$ nm, and $+11.2 \pm 3.18$ mV and $+7.27 \pm 4.34$ mV, for amine-PBAE and acrylate-PBAE, respectively. In this study, quaternization was used as a method to enhance the positive charge density of PBAE, thereby promoting its siRNA binding efficiency. The results suggested that the nanoparticles were positively charged when the weight ratio of PBAEQAS/siRNA was higher than 25:1. PBAEQAS could condense siRNA into nanoparticles with the size of about 100 nm when the weight ratio of PBAEQAS/siRNA was higher than 150 and the surface charge of the nanoparticles was about $+20$ mV. A difference in the binding efficiency of siRNA could be observed between PBAEQASs with different terminal groups. As shown in Figure 2D, the size of amine-terminated PBAEQAS was smaller and the surface had a higher positive charge compared to acrylate-terminated PBAEQAS. Thus, amine-terminated PBAEQAS had a more suitable size and potential value for siRNA delivery than acrylate-terminated PBAEQAS.

The retardation assay and the protection test against RNase A were detected by agarose gel electrophoresis. When the weight ratio (polymer/siRNA, w/w) was above 100, the siRNA loaded in amine-PBAEQAS was almost retained within the gel-loading well compared with amine-PBAE/siRNA nanoparticles (Figure 2A). The siRNA was totally retained within the gel well when the weight ratio of acrylate-PBAEQAS/siRNA nanoparticles was higher than 50 (Figure

![Figure 4](https://example.com/figure4.png)

Figure 4. Fluorescence images of A549 cells showing cellular uptake after incubation with FAM-siRNA-loaded nanoparticles for 6 h: (A) with amine-PBAE/siRNA nanoparticles, (B) with amine-PBAEQAS/siRNA nanoparticles, (C) with acrylate-PBAE/siRNA nanoparticles, and (D) with acrylate-PBAEQAS/siRNA nanoparticles. Images show fluorescent overlay of siRNA (green, FAM-labeled) and nuclear (blue, Hoechst33342-stained).
concentration of polymers increased. Thus, it could be respectively, the cell cytotoxicity increased obviously as the nanoparticles. The decrease of the viability may be attributed PBAEQAS/siNC nanoparticles compared with PBAE/siNC was observed to decrease when the cells were incubated with was no signifi
can't correlation between polymer molecular
cationic polymers, the siRNA loaded in polymer/siRNA degraded in 5 min. However, with the protection of the cationic polymers, the siRNA loaded in polymer/siRNA polyplexes was subject to less RNase degradation (Figure 3C). The results indicated that PBAEQAS could not only effec
tively bind with siRNA but also offer better protection of siRNA from degradation than PBAE.

3.3. Cytotoxicity Assay. Cytotoxicity assay of the polymer/siRNA was performed to select the most optimal formulation based on uptake and transfection of nanoparticles in A549 cells. Initially, PBAE/siNC nanoparticles and PBAEQAS/siNC nanoparticles formed with various weight ratios of polymer and siRNA were tested to select the best vector. As shown in Figure 3A, the cell growth inhibition rate of PEI, 25 kDa, was much higher than those of all other polymers at each concentration. PBAEs have higher cell viability than PBAEQAS. Eltoukhy et al. had proved that there was no significant correlation between polymer molecular weight and toxicity,42 so we hypothesized that the structure of polymers affects their cytotoxicity. The viability of A549 cells was observed to decrease when the cells were incubated with PBAEQAS/siNC nanoparticles compared with PBAE/siNC nanoparticles. The decrease of the viability may be attributed to the increase of positive charge of the polymers.43 The cell viability was over 60% when A549 cells were treated with the amine-terminated polymers for 48 h, even at a high concentration of 100 μg/mL, while the cell viability of acrylate-terminated polymers was lower, as shown in Figure 3A. The viability of acrylate-terminated PBAEQAS/siNC nanoparticles was close to that of PEI at 100 μg/mL. Respectively, the cell cytotoxicity increased obviously as the concentration of polymers increased. Thus, it could be concluded that the concentration had a certain influence on cytotoxicity, and the concentration of the nanoparticles should not exceed 100 μg/mL for amine-PBAEQAS/siRNA nanoparticles and 50 μg/mL for acrylate-PBAEQAS/siRNA nanoparticles in subsequent transfection experiments.

3.4. Cellular Uptake and Mechanisms. As shown in Figure 3C, the mean fluorescence intensity of the cells after incubation with siRNA-loaded nanoparticles changed at different times. The results suggested that the highest intracellular siRNA delivery efficiency occurred at 6 h, because it took a certain time for the nanoparticles to be internalized into the cell. The percentage of cells that internalized FAM-labeled siRNA and their mean fluorescence intensity (MFI) were measured by flow cytometry analysis to evaluate the cellular uptake efficiency. As can be seen in Figure 3B, the quantitative internalization of acrylate-PBAEQAS was close to that of amine-PBAEQAS, but the cellular uptake of all kinds of PBAEQAS was higher than that of PBAE, and the uptake of acrylate-PBAE was higher than that of amine-PBAE. As can be seen in Figure 4, the qualitative fluorescence image reveals that cellular uptake of acrylate-PBAEQAS/siRNA is stronger compared with that of amine-PBAEQAS/siRNA.

Nanoparticles appear to enter the cell via charge adsorptive endocytosis initiated by the reaction between positively charged surface and negatively charged cell membranes; that is to say, the increase of positive charge density can improve the cellular uptake of nanoparticles.44 The polymers exhibit more positive charge after quaternization; thus, PBAEQAS/siRNA nanoparticles had higher cellular uptake efficiency than PBAE/siRNA nanoparticles. As the structure of the polymers affects the efficiency of cellular uptake,45 we hypothesize that the QA groups of PBAEQAS may also improve the cellular uptake.

The uptake of amine-PBAEQAS/siRNA polyplexes resulted in a same transfection rate as with acrylate-PBAEQAS/siRNA polyplexes, possibly due to endocytosis pathways or rate-limiting downstream steps.46 The results of the cellular uptake of polymers showed that further investigation would be necessary to clarify the cellular uptake mechanism. The

Figure 5. (A) Flow cytometry analysis of A549 cells for apoptosis after treatment with different formulations (n = 3). (B) siSurvivin-mediated silencing of A549 assessed by using qRT-PCR (ΔΔCq). GAPDH was set as endogenous reference gene (n = 3).
endocytotic mechanisms include clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin-independent endocytosis, caveolin-independent endocytosis, and macropinocytosis. Clathrin-mediated endocytosis is destined for a lysosomal compartment, while a carrier internalized via the pathway of caveole-mediated endocytosis could avoid the fate of lysosomes. The endocytosis pathway was dependent on the particle size, shape, chemical structure, etc. 47−49

To investigate the endocytosis pathways of the polyplexes into the cell, endocytic inhibitors were applied. As shown in Figure 3D, the results revealed significant inhibitory effects of sodium azide and 4 °C incubation, which indicated that it was internalized into A549 cells by energy-dependent endocytosis. When the cells were incubated with chlorpromazine and hypertonic sucrose (clathrin-mediated endocytosis inhibitors), they exhibited comparatively low uptake levels compared with those treated with filipin and genstein (caveole-mediated endocytosis inhibitors). The fact that uptake was only decreased to 80% when the cells were treated with amiloride indicated that the cellular entry of polyplexes was relevant to macropinocytosis, but it was not the main pathway. Thus, it can be concluded that the cellular uptake of polyplexes was mainly based on clathrin-mediated and caveola-dependent endocytosis, because the chain structure of the polymers may affect the endocytosis pathway. 50

3.5. Apoptosis of A549 Cells. Survivin is one of the inhibitors of apoptosis protein, 51 which can promote the proliferation of tumor cells, and the anti-proliferation effect of siSurvivin probably induces apoptosis of tumor cells. As illustrated in Figure 5A, the apoptosis assay results were obtained via an apoptosis assay kit (Annexin V-FITC/PI). After 24 h of transfection, there were almost no apoptotic cells in the groups of naked siSurvivin, siNC-loaded polyplexes. However, acrylate-PBAEQAS/siSurvivin polyplex and amine-PBAEQAS polyplex induced (32.79 ± 0.69)% and (36.70 ± 2.12)% apoptotic cells, compared with the control cells, respectively (n = 3). As discussed previously, quaternization of PBAE increased the positive charge density in the polymer, and thus increased the uptake ability of siRNA. 50 As shown in Figure 3, the quantitative internalization of acrylate-PBAEQAS was close to that of amine-PBAEQAS, but the viability of A549 cells was close to that of amine-PBAEQAS, but the viability of A549 cells than acrylate-PBAEQAS/siSurvivin nanoparticles. Consequently, amine-PBAEQAS/siSurvivin nanoparticles resulted in higher apoptosis percentages of A549 cells than acrylate-PBAEQAS/siSurvivin nanoparticles.

3.6. Gene Silencing Assay. In this study, anti-survivin siRNA was employed to complex with cationic polymer and delivered into A549 cells. The silencing efficiency of polymer/siSurvivin nanoparticles was evaluated by qRT-PCR. The survivin expression in A549 cells is shown in Figure 5B. The acrylate-PBAEQAS/siSurvivin nanoparticles (containing 50 nM siSurvivin) and amine-PBAEQAS/siRNA nanoparticles (containing 50 nM siSurvivin) reduced the level of survivin mRNA to (49.23 ± 2.24)% (p < 0.001) and (41.08 ± 6.46)% (p < 0.001), respectively. Polymer/siNC nanoparticles were set as the negative control. These results suggest that quaternized PBAEQAS has certain potential as gene delivery vector.

4. CONCLUSION

In this study, a novel gene delivery system based on PBAEQAS was successfully prepared. The polymer can condense siRNA into nanoparticles with the size of ∼100 nm. The zeta potential was about +20 mV. The siRNA retardation assay showed that siRNA was effectively condensed into nanoparticles because of the enhanced positive charge density. Cell cytotoxicity assay showed PBAEQAS had lower cytotoxicity than PEI. The cellular uptake of PBAEQAS/siRNA nanoparticles was mainly based on clathrin-mediated and caveole-dependent endocytosis. PBAEQAS/siSurvivin polyplexes had significant gene silencing efficiency and high apoptosis of A549 cells. Collectively, the results of this study demonstrate that quaternization of PBAE can be a promising way to improve the transfection efficiency of low-molecular-weight PBAE. PBAEQAS developed in this study shows its potential as a promising siRNA vector for cancer gene therapy. In the future, we will focus on combining PBAEQAS with other materials to expand its application.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.8b00549. Figure S1, GPC traces of amine-PBAE, amine-PBAEQAS, acrylate-PBAE, and acrylate-PBAEQAS (PDF)

AUTHOR INFORMATION

Corresponding Author

*Tel.: +8613851713608. Fax: +862583271355. E-mail: tanygue@cpu.edu.cn.

ORCID

Yue Tang: 0000-0001-9915-5499

Author Contributions

These authors contributed equally to this work and should be regarded as co-first authors.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the third Jiangsu Overseas Research & Training Program for University Prominent Young & Middle-aged Teachers and Presidents, the College Students Innovation Project for the R&D of Novel Drugs [No. J1310032]. We would like to thank the cell and molecular biology experiment platform of China Pharmaceutical University for the assistance with relevant test items.

ABBREVIATIONS

PBAE, poly(β-amino ester); PLGA, poly(lactic-co-glycolic acid); PAMAM, poly(amine); PLL, poly(l-lysine); PEI, poly(ethyleneimine); MW, molecular weight; PEG, poly(ethylene glycol); PBAEQAS, PBAE quaternary amine; PAMAM, poly(amidoamine); PLL, poly( L-lysine); MW, molecular weight; PEG, poly(ethylene glycol); PBAEQAS, PBAE quaternary amine; siSurvivin, Survivin siRNA; RNase A, ribonuclease A; MT, thiazoyl blue tetrazolium bromide (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide); qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; PI, propidium iodide; siNC, negative control siRNA; ATCC, American Type Culture Collection; FBS, fetal bovine serum; QA, quaternary ammonium; PGE, gel permeation chromatography; DLS, dynamic light scattering; MFI, mean fluorescence intensity

DOI: 10.1021/acs.molpharmaceut.8b00549

Mol. Pharmaceutics 2018, 15, 4558−4567
REFERENCES


