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Structural Mediation on Polycation Nanoparticles by Sulfadiazine to Enhance DNA Transfection Efficiency and Reduce Toxicity

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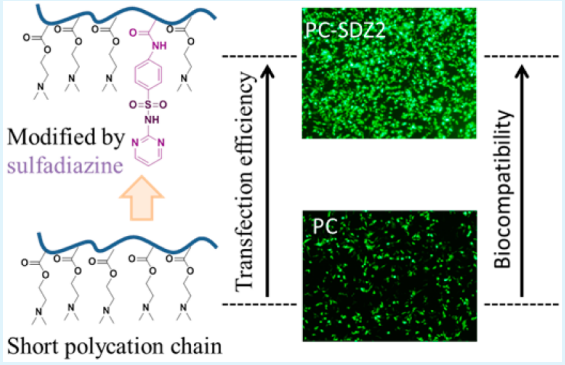
Supporting Information

ABSTRACT: Reducing the toxicity while maintaining high transfection efficiency is an important issue for cationic polymers as gene carriers in clinical application. In this paper, a new zwitterionic copolymer, polycaprolactone-*g*-poly(dimethylaminoethyl methyacrylate-*co*-sulfadiazine methacrylate) (PC-SDZ) with unique pH-sensitivity, was designed and prepared. The incorporation of sulfadiazine into poly(dimethylaminoethyl methacrylate) (PDMAEMA) chains successfully mediates the surface properties including compacter shell structure, lower density of positive charges, stronger proton buffer capability, and enhanced hydrophobicity, which lead to reduction in toxicity and enhancements in stability, cellular uptake, endosome escape, and transfection efficiency for the PC-SDZ2 nanoparticles (NPs)/DNA complexes. Excellent transfection efficiency at the optimal N/P ratio of 10 was observed for PC-SDZ2 NPs/DNA complexes, which was higher than that of the commercial reagent-branched polyethylenimine (PEI). The cytotoxicity was evaluated by CCK8 measurement, and the results showed significant reduction in cytotoxicity even at high concentration of complexes after sulfadiazine modification. Therefore, this work may demonstrate a new way of structural mediation of cationic polymer carriers for gene delivery with high efficiency and low toxicity.



KEYWORDS: *DNA delivery, sulfadiazine, pH sensitivity, transfection efficiency, cytotoxicity*

INTRODUCTION

The controlled gene delivery based on different kinds of nonviral vectors has been raising much concern during the past several decades both in vitro and in vivo.[1−4] Due to its negative charge and poor stability in the bloodstream, nucleic acid itself cannot be delivered to pathogenic sites and transported into cells. Therefore, appropriate vehicles for gene delivery are required. Up to now, lots of materials with varied structures have been exploited including liposome, polymer, and inorganic materials.[5] Among these systems, polycations like polyethylenimine (PEI),[6] poly(dimethylaminoethyl methacrylate) (PDMAEMA),[7] polyamidoamine (PAMAM),[8] and poly(*β*-amino ester) (PBAEs)[9] show good potential because of the convenience in structural modification, the efficiency in condensing nucleic acids, less immunogenicity, and relatively low cost.[10] Although the cationic charge of polycation provides strong membrane binding of delivery vehicles and facilitates cellular uptake and transfection of the gene cargos, it meanwhile causes severe associated toxicity.[11] Generally, increasing the length of polycation chains is an effective way to improve the transfection efficiency, but that is often accompanied by increased toxicity due to metabolic problems of the nonbiodegradability of polycation.[12,13] Therefore, it is of particular importance to balance the efficiency and toxicity in the design of nonviral vectors.

To improve the gene transfection efficiency and reduce toxicity, the courses of gene delivery including gene condensation, cellular uptake, endosomal escape, and gene unloading have been considered one by one.[14−17] For instance, a series of polycations like poly[(3-morpholinopropyl)-aspartamide] (PMPA),[18] poly(Diisopropylamino) (PDPA),[19] and poly(2-(dimethylamino)ethyl methacrylate) (PDMAE-

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MA)[20,21] have been introduced to enhance the escape capability from endosome or lysosome. However, too much positive charge may cause toxicity and block the efficient release of nucleic acid from the nanoparticles (NPs). For the safety of carriers, the use of poly(ethylene glycol) (PEG) as biocompatible shell is one straightforward approach to decrease the toxicity but usually leads to lower transfection efficiency. Multiple self-assembly is another important strategy which has been widely used to reduce toxicity along with function modification.[22−24] Hyaluronic acid, an important polyanion, has been introduced to reduce toxicity and target the CD44 receptor for enhanced transfection efficiency via coating the PCL-*g*-PDMAEMA NPs, and the ternary complexes have performed excellent transfection in vitro and in vivo.[25] Except for those, it has been reported that the incorporation of some polyanion into the polycation can promote the proton sponge effect by capturing the proton along with cation by anion. Due to the responsible behaviors of poly(methacrylic acid) (PMAA), polybutadiene-*b*-poly(methacrylicacid)-*b*-poly(2-(dimethylamino)ethyl methacrylate) (BMAAD) has been reported to generate a surface reminiscent to that of certain viruses and be capable of undergoing pH-dependent changes in charge stoichiometry, which exhibits BMAAD was a kind of efficient and safe gene carrier especially on human leukemia cells.[26] Oligomeric sulfonamides (OSAs) have been explored as a tool for the effective endosomal release of polyplexes. They perform proton-buffering regions and pH-dependent solubility transitions within the endosomal pH range, which benefits endosomal-membrane destabilization for enhanced transfection efficiency.[27]

Our previous research on DNA carriers based on amphiphilic copolymer PCL-*g*-PDMAEMA has verified that these carriers performed high transfection efficiency.[7,28] However, long PDMAEMA chains always cause high toxicity due to high positive charge density on the NPs, while short PDMAEMA chains can lead to the lower transfection efficiency, which makes it necessary to overcome the inconsistency. Therefore, the sulfadiazine group with pK_a of about pH 6.5 was incorporated into short PDMAEMA chains to affect or change the properties of NPs and its function as DNA carriers because the sulfadiazine group possesses the properties of reversible process of ionization from pH 5.0 (endosomal environment) to pH 7.4 (physiological environment). At pH 5.0 or under pH 6.5, the sulfadiazine group becomes hydrophobic by deionization, and the polycation NPs modified by sulfadiazine may become more compact, which can prevent the DNA dropping out from the DNA/NPs complexes in the endosome instead of the cytoplasm. The enhanced hydrophobicity may also benefit the cellular uptake of NPs. At pH 7.4 or above pH 6.5, the sulfadiazine group carries negative charges and may shield some positive charges produced by protonation of DMAEMA, eventually reducing the toxicity of polycation NPs due to lower positive charge density. Herein, a pH-sensitive zwitterionic copolymer, poly(ε-caprolactone)-*g*-poly-(dimethylamino ethyl methacrylate-*co*-sulfadiazine ethyl methacrylate) (PC-SDZ), was prepared. Physicochemical properties of the NPs or NPs/DNA complexes and in vitro gene transfection, intracellular uptake, and cytotoxicity were evaluated by using a combination of different analytical techniques including dynamic light scattering, transmission electron microscopy, contact angle testing, inversion fluorescence microscopy, a fluorescence-activated cell sorter, and confocal laser scanning microscopy.

EXPERIMENTAL SECTION

Materials. γ-(2-Bromo-2-methylpropionate)-ε-caprolactone (BMPCL) was synthesized as reported previously.[29,30] Benzyl alcohol, ε-caprolactone (ε-CL), stannous octanoate ($Sn(Oct)_2$), 2-(dimethylamino)ethyl methacrylate (DMAEMA), copper(I) bromide, pentamethyl diehylenetrihylenetriamine (PMDETA), sulfadiazine (SDZ), and methacryloyl chloride were purchased from Sigma-Aldrich without further purification. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, Lysotracker Red DND, and PEI (branched, 25 kDa) were purchased from Invitrogen Corp (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Minhai Corporation (Beijing, China). Cell proliferation reagent CCK8 was purchased from Life Corp. (Basel, Switzerland). FAM-labeled oligo-DNA (5′-FAM-ATT AAC CCT CAC TAA AGG GA-3′) and pmirGLO were purchased from Generay Biotech Co. Ltd. (Beijing, China).

Synthesis of PC-SDZ. The methacryloylated SDZ was synthesized as described previously.[31] In brief, preweighed sulfadiazine (10 mmol) was dissolved in 40 mL aqueous/acetone mixture solvent (NaOH (0.5 N; aqueous (aq.))/acetone = 1:1 (v/v)). Then, methacryloyl chloride (10 mmol) was slowly added into the sulfadiazine solution for 1 h at 0−5 °C under vigorous stirring. The precipitated product was filtered and washed twice by water, then recrystallized in methanol twice for purification, dried, and stored in vacuum for the following use.

The macroinitiator P(CL-*co*-BMPCL) was also synthesized as described previously.[28] Preweighed benzyl alcohol (0.3 mmol), BMPCL (0.9 mmol), ε-CL (15 mmol) monomer, and stannous octoate (1 wt %) were quickly added to a 10 mL dried Schlenk flask and vacuumed for about 20 min. Then, the reactor was sealed in a nitrogen atmosphere. After constant stirring at 140 °C for 12 h, the product was first dissolved in dichloromethane (DCM) (2 mL) and then added dropwise into an excessively cold diethyl ether (80 mL) to obtain P(CL-*co*-BMPCL).

To be specific, preweighed P(CL-*co*-BMPCL) (0.05 mmol), DMAEMA(10 mmol), PMDETA, and copper(I) bromide were quickly added to a 10 mL dried Schlenk flask with 2 mL of DMSO and then vacuumed and sealed in a nitrogen atmosphere for three times; after constant stirring for 48 h at 60 °C, the polymer solution was purified by dialysis against double-distilled water for 48 h and then freeze-dried to obtain the product PC. The sulfadiazine-modified PC copolymer (PC-SDZ) was also prepared by ATRP in one-pot as mentioned above. Specifically, after constant stirring for 48 h at 60 °C, the polymer solution was purified by dialysis against DMSO for 24 h (for removing the unreacted methacryloylated sulfadiazine) and against double-distilled water for 48 h and then freeze-dried to obtain the product PC-SDZ.

Polymer Characterization. The molecular weights of polymers were determined by gel permeation chromatography (GPC), and chemical structure was analyzed by NMR spectroscopy. The gel permeation chromatography system (GPC, Agilent 1100, Santa Clara, CA) was used to measure the molecular weight and molecular weight distribution of polymers. Tetrahydrofuran (THF) was used as the eluent at a low flow rate of 1.0 mL/min. Monodispersed polystyrene standards were used to generate the calibration curve. ^{1}H NMR spectra were recorded on a Varian INOVA 500 MHz NMR machine, using $CDCl_3$ and $(CD_3)_2SO$ as solvent and tetramethylsilane (TMS) as internal standard.

Preparation of PC-SDZ NPs and NPs/DNA Complexes. The charge ratio (N/P) of binary complexes was indicated as the moles ratio of moles of the tertiary amine groups (N) on PC or PC-SDZ to the phosphate groups (P) on plasmid. The NPs were prepared by nanoprecipitation technology. Briefly, preweighed PC or PC-SDZ copolymers were dissolved in 1 mL THF and then added to 10 mL double-distilled water dropwise, separately. After stirring at room temperature overnight to remove THF, the final volume was adjusted to 10 mL by adding doubled-distilled water and the NPs at a concentration of 10 nmol N μL^{-1} were obtained. The NPs/DNA complexes were prepared by mixing the NPs dispersions with the

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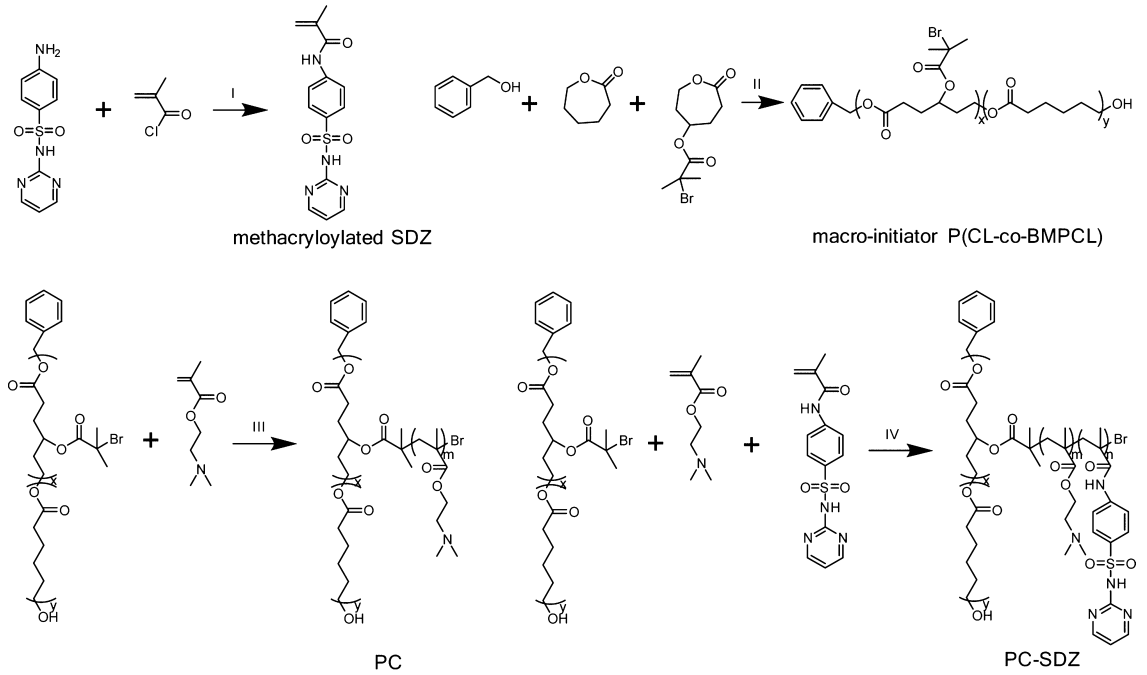
Scheme 1. Synthesis Routes of Methacryloylated SDZ, P(CL-*co*-BMPCL), PC, and PC-SDZ[a]



[a](I) NaOH (0.5 N; aqueous (aq.))/acetone as solvent, 0−5 °C, 1 h; (II) $Sn(Oct)_2$ as catalyst, 130 °C, 12 h; (III) CuBr/PMDETA as catalyst, DMSO as solvent, 60 °C, 48 h; (IV) CuBr/PMDETA as catalyst, DMSO as solvent, 60 °C, 48 h.

plasmid DNA (1 mg/50 mL) TB solution, which were incubated for 20 min for property characterization and the biology experiment.

Characterization of PC-SDZ NPs. The particle size and zeta potentials of PC-SDZ NPs in aqueous solution at different pH values (5.0, 6.0, 6.5, 7.0, 8.0, and 9.0) were determined in this work using a Zetasizer 3000HS (Malvern Instrument, Inc., Worcestershire, UK) at a wavelength of 677 nm with a constant angle of 90° at room temperature. The concentration of all NPs was 5 nmol N μL^{-1}. The morphology of PC, PC-SDZ1, and PC-SDZ NPs was observed using JEM-100CX II (Holland). Sample was prepared in water and dropped on a copper TEM grid with a carbon film and air-dried at room temperature.

Water Contact Angle (WCA) Test. The hydrophobicity of copolymers was investigated through testing the water contact angle of copolymer films. The films were prepared by the solvent evaporation method, and the water contact angle was measured using a drop shape analysis system DSA 10 MK2 (Kruss) at room temperature.

Agarose Gel Electrophoresis Retardation Assay. In this study, agarose gel electrophoresis was used to assess DNA-binding ability of the PC and PC-SDZ NPs at pH 7.4 and 5.0. Complexes (containing 0.5 mg DNA) at various N/P ratios were mixed with 4 μL 6× loading buffer and loaded into a 0.8 wt % agarose gel containing 0.5 mg/mL ethidium bromide. Electrophoresis was run in 1 TAE buffer at 110 V for 40 min. DNA retardation was analyzed using a UV illuminator (Bio-Rad, Hercules, CA) to show the band of the DNA.

Acid−Base Titration Test. The buffering capacities of the polymers were determined through acid−base titration. Ten mL of prepared NPs dispersion with a concentration of 10 nmol N μL^{-1} was diluted to 20 mL with 0.2 M NaCl solution. The pH value of polymer solutions was set to pH 4 using 0.2 M HCl and then titrated against 0.2 M NaOH. The pH values of the solutions were measured after each addition using a PHS-2C pH meter (Lida Instruments, China).

Cell Culture. HEK 293T human normal cell, MCF7 human breast cancer cells, and HeLa human cervical cancer cells were selected as tool cells to assess the transfection or toxicity of gene carriers in this experiment. The DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycinin were utilized as the cell culture medium. Cells were cultivated at 37 °C with 5% CO^2 and 95% humidity. Before the experiment, the cells were precultured until confluence was reached to 70−80%.

Green Fluorescent Protein Assay. Before gene transfection, HEK 293T cells were seeded in 24-well plates at an initial density of 2 × 10^4 cells per well in 0.5 mL growth medium and incubated for 18−20 h to achieve 70−80% confluence. The medium was replaced by 0.5 mL DMEM medium containing NPs/pEGFP-N1 complexes (containing 1 μg plasmid) at various N/P ratios (5, 10, 15, and 20) and continuously incubated for 4 h. Serum-free medium was then replaced by complete culture medium, followed by incubation for 24 h. Transfection experiments were performed on two wells per sample. The cells were directly observed using an inverted microscope (Eclipse 80i, Nikon, Japan). After observation by microscopy, the cells were then washed three times with 1 mL PBS to remove residual free complexes, suspended in 300 mL PBS and subsequently introduced into a BD FACS Verse flow cytometer (Becton Dickinson, San Jose 263, CA).

Luciferase Assay. Prior to transfection, HEK 293T cells were seeded in 48-well plates at an initial density of 1.5 × 10^4 cells per well in 0.25 mL growth medium and incubated for 18−20 h to achieve 70−80% confluence. The medium was replaced by 0.25 mL DMEM medium containing NP/pmirGLO complexes (containing 0.6 μg of plasmid) at various N/P ratios and continuously incubated for 4 h. Serum-free medium was then replaced by complete culture medium, followed by incubation for 24 h. The medium was removed, and the cells were rinsed twice with PBS. 0.2 mL 1× Reporter Lysis Buffer (Promega, Madison, WI) was added to each well to lyse the cells. After the cell lysate was centrifuged for 10 min at 12,000 r.p.m. at 4 °C, the supernatant was collected for luminescence measurements. Following the manufacturer's protocol for the dual luciferase reporter gene assay (Promega, Madison, WI), the relative light unit (RLU) ratio between firefly luciferase and ranilla luciferase was measured with a chemiluminometer (Autolumat LB953, EG&G, Berthold, Germany).

Cell Uptake Studies. For the cellular uptake of complexes studies, HEK 293T cells were plated in 24-well plates at 2 × 10^4 cells per well for 24 h before the experiment. The cells were treated with NP/DNA complexes containing 200 pmol FAM-labeled oligo-DNA and incubated for 4 h at 37 °C. The cells were then washed three times with 1 mL PBS to remove residual free complexes, suspended in 400

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mL PBS, and subsequently introduced into a BD FACS Verse flow cytometer (Becton Dickinson, San Jose 263 CA).

Confocal Laser Scanning Microscope (CLSM) Assay. HEK 293T cells were plated in 12-well plates with glass coverslips at the bottom at 2×10^5 cells per well for 24 h before transfection. After the culture medium was changed to DMEM with free serum, the incubated complex solutions containing 200 pmol FAM-labeled oligo-DNA (N/P = 10) was added to each plate. After 4 h incubation at 37 °C in a 5% CO_2 humidified atmosphere, the transfection solutions were aspirated and substituted by complete culture. Each well was stained with Lysotracker red to indicate endosomal/lysosome organelles and was stained with DAPI to indicate the cell nucleus, then, the wells were washed with PBS three times 30 min later. The intracellular distribution was observed by confocal microscopy (TCS SP5, Leica, Germany). Imaging processing programs were coded in Interactive Data Language.

Cell Viability Assay. The cytotoxicity of the NPs/DNA complexes against HEK 293T cells and MCF7 cells was assessed using the CCK8 assay. Cells were seeded at 1.0×10^3 cells/well in 96-well plates and cultured in serum-containing media for 24 h as previously described. Then, the medium was replaced with DMEM medium (100 μL per well), in which NPs/DNA complexes were added at the DNA final concentration of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 μg/well, respectively. After incubation at 37 °C for 4 h, the medium was changed to serum-containing DMEM and cells were further cultured for 24 h before viability assessment. Subsequently, 20 μL fresh DMEM solutions containing 8 μL CCK8 reagents were added to each well and incubated for another 1 h. Finally, the absorbance was measured at 490 nm by a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated according to the following equation:

$$\text{cell viability}\,(\%) = (A_{\text{sample}}/A_{\text{control}}) \times 100\%$$

where A_{sample} was the absorbance of the complex treated cells and A_{control} was the absorbance of the untreated cells. Each experiment was done in quadruplicate. Results were represented as percentage viability of control cells that did not receive complex treatment.

RESULTS AND DISCUSSION

Synthesis and Characterization of PC-SDZ. In our study, PC and PC-SDZ was synthesized by combining ring-opening polymerization (ROP) and atom transfer radical polymerization (ATRP) techniques. As shown in Scheme 1(I) and Figure S1, Supporting Information, SDZ is methacryloylated via amide reaction for the next polymerization and the characteristic peaks at 1.92 ppm for $-CH_3$(3H) and 8.5 ppm for $-C_4H_3N_2$(2H) in the ^{1}H NMR spectrum are clearly displayed and prove the successful preparation of the comonomer. The characteristic peaks of benzyl ring group moieties around 7.1 ppm, PCL moieties at 1.36 ppm, and BMPCL moieties at 1.92 ppm are observed in the ^{1}H NMR spectrum shown in Figure S2, Supporting Information, indicating that the P(CL-*co*-BMPCL) copolymer as macromolecular initiator is obtained by ROP of ε-caprolactone and BMPCL, Scheme 1(II). The mean number of BMPCL units in the copolymer is 2.9, and the Mn of P(CL-*co*-BMPCL) is 4.9×10^3 g/mol. PC or PC-SDZ is prepared by ATRP with DMAEMA or DMAEMA and methacryloylated SDZ as shown in Scheme 1(III, IV) and Figure 1. The number of PDMAEMA units is estimated by comparing the intensities of signals at 2.58 and 2.29−2.32 ppm which belong to PDMAEMA moieties and PCL moieties, respectively (Figure 1A). The number of SDZ units is estimated by comparing the intensities of signals at 8.44−8.48 and 2.32−2.34 ppm which belong to SDZ moieties and PCL moieties, respectively (Figure 1B,C). The details of PC and PC-SDZ are shown in Table 1.

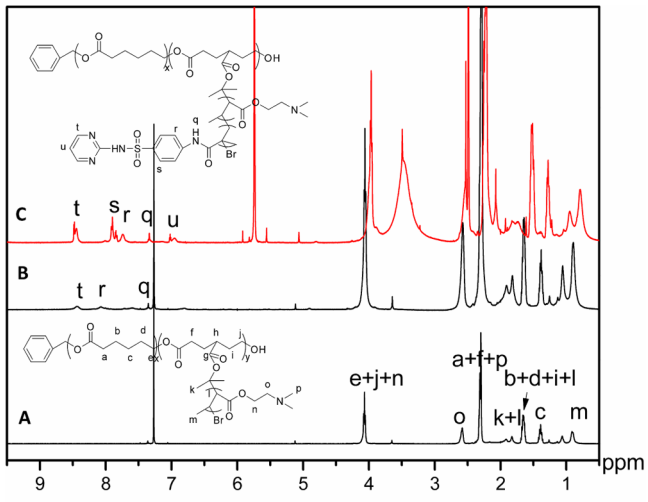

Figure 1. ^{1}H NMR spectroscopy of PC in $CDCl_3$ (A), PC-SDZ in $CDCl_3$ (B), and PC-SDZ in $(CD_3)_2SO$ (C).

Table 1. Structure Detail of Copolymer

sample	DP[a] (DMAEMA)	DP[a] (SDZ)	M_n[b] (kDa)	M_w/M_n[c]
PC	15		13.6	1.31
PC-SDZ1	15	1.4	14.9	1.28
PC-SDZ2	15	3.1	16.5	1.30

[a]The unit numbers for DMAEMA and SDZ per graft chain were determined by ^{1}H NMR. [b]The molecular weight was calculated by ^{1}H NMR. [c]The molecular weight distribution was determined by GPC.

Characterization of PC-SDZ NPs. The amphiphilic copolymer can undergo self-assembly into core−shell structure NPs in aqueous solution. Different core or shell moieties often have significant effect on the property and application of NPs. Here, DLS and zeta potential measurements were applied to investigate the effect of the sulfadiazine group in the PDMAEMA chain on the size and surface zeta potential in the self-assembly process as shown in Figure 2. Figure 2A shows that the sizes of PC, PC-SDZ1, and PC-SDZ2 NPs increase when the pH values decrease from 9 to 5. This is because the gradually promoted protonation of PDMAEMA segments caused enhanced water-solubility of PDMAEMA chains and enhanced electrostatic repulsion between polycation chains, which is similar to previous studies.[32,33] It should be noted that PC, PC-SDZ1, PC-SDZ2, and NPs present different size changes with about a 60, 30, and 20 nm increase, respectively. Thus, the existence of SDZ moieties helps the NPs keep a more compact structure in acidic circumstances. Meanwhile, the existence of SDZ moieties also lowers the positive zeta potential of the NPs as shown in Figure 2B, which has a benefit to reduce toxicity. A conversion from positive to negative charge upon the pH value changing from 8 to 9 is observed in the case of PC-SDZ1 and PC-SDZ2 NPs, which is caused by the fact that the degree of ionization of the SDZ moiety exceeded that of the PDMAEMA moiety in or over the pH scope. Obviously, the contribution of sulfadiazine groups can cause a lower pK_a value and the process of protonation−deprotonation or ionization−deionization between DMAEMA and sulfadiazine balances the charge and size responses to the varied pH values. Interestingly, the PC-SDZ1 and PC-SDZ2 NPs observed by TEM have some chipped edges compared to the PC NPs (Figure 2C). This may be contributed by the

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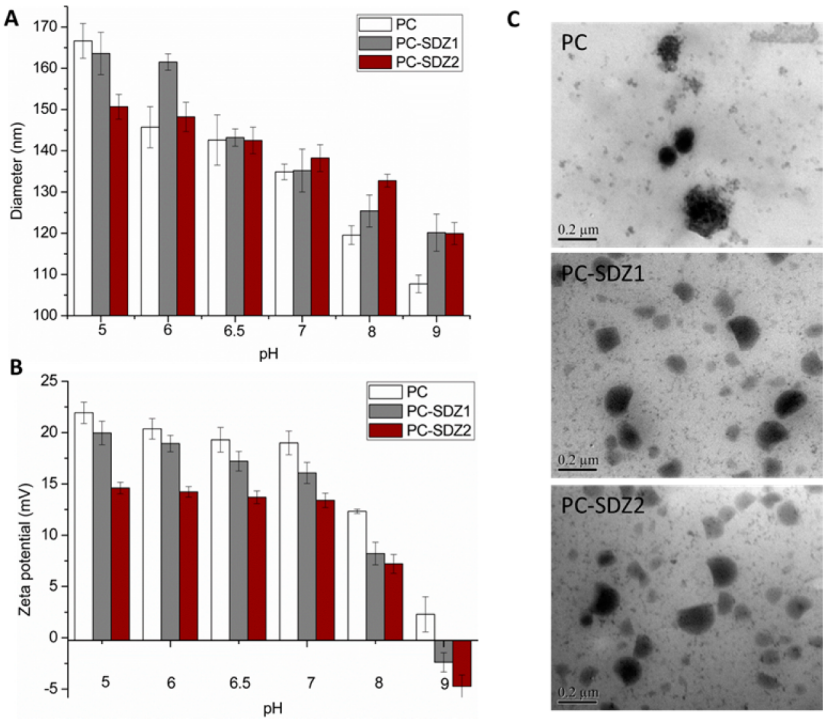

Figure 2. Hydrodynamic diameters (A) and zeta potential (B) of NPs at different pH environments and TEM images of NPs (C). The samples for TEM were prepared at pH 6.5.

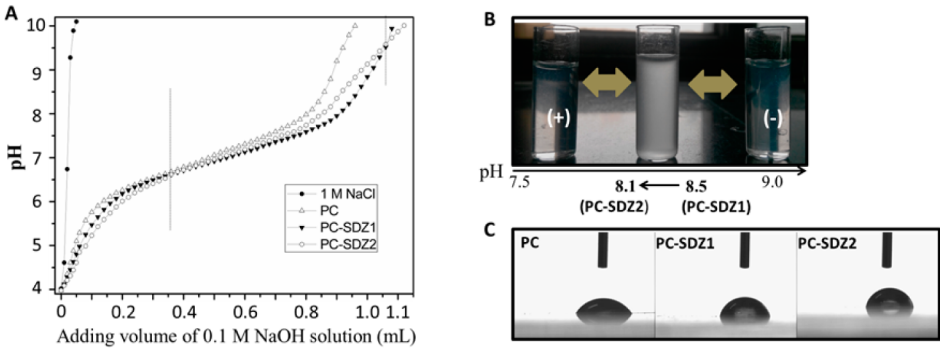

Figure 3. (A) Acid−base titration curves for selected polymers normalized for 1 M aqueous NaCl. Measurements were taken using a pH meter. (B) pH-dependent phase behavior of PC-SDZ2 NPs. (C) Water contact angles of PC, PC-SDZ1, and PC-SDZ2 NPs at the pH of 6.5.

interaction between SDZ and DMAEMA units. It has been reported that the presence of two oppositely charged polyelectrolyte structures within NPs may lead to the formation of intramicellar interpolyelectrolyte complexes in the shell of the particles.[26,33]

By now, a question should be raised that if SDZ moieties affect the ability of polycation to buffer the acidic endosome compartment due to the decrease in positive charge, further affecting the escape from endosomes. To assess proton buffer ability of the NPs, the proton buffer curves were determined by acid−base titration. As shown in Figure 3A, the proton buffering capability of PC-SDZ1 and PC-SDZ2 is similar or even slightly higher than PC around 5.0−5.5. The pH-dependent phase behaviors of PC-SDZ1 NPs and PC-SDZ2 NPs are shown in Figure 3B. For PC-SDZ2 NPs, under pH 8.1, the zeta potential of NPs is positive. Above pH 8.1, the zeta potential of NPs is negative. At pH 8.1, the NPs become hydrophobic because of the isoelectric point state without any charges. Also, the isoelectric points are reduced with the sulfadiazine content increasing, which indicates the effect of the structures or properties of NPs by the sulfadiazine group.

Considering that the hydrophobicity of NPs shell is also an important issue to affect the gene delivery efficiency,[34−36] the water contact angle tests were used to investigate the effect of sulfadiazine modification to the hydrophobicity of the NPs shell. Obviously, as shown in Figure 3C, sulfadiazine modification for sure increases the hydrophobicity of NPs shell due to the electrostatic interaction between the DMAEMA group and the sulfadiazine group and discharge of sulfadiazine.[37−39]

DNA Loading Capability of the NPs. The successful gene delivery requires that the carriers have good gene loading capability including efficient loading, avoiding the premature unloading or enzymolysis in the endosome. As we know, the pH value of plasma in normal blood circulation is 7.4 while that in endosome or lysosome is around 5.0−5.5. In order to investigate how the sulfadiazine modification affects the nucleic acids loading capability of the polycation NPs, pH 5.0 and 7.4

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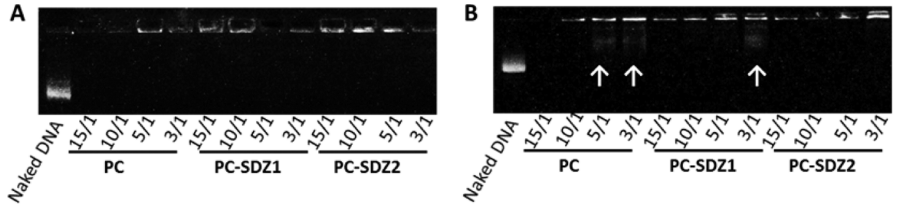

Figure 4. Agarose gel electrophoresis retardation assay of the NPs at various N/P ratios of NP/DNA complexes at different pH values of 7.4 (A) and 5.0 (B).

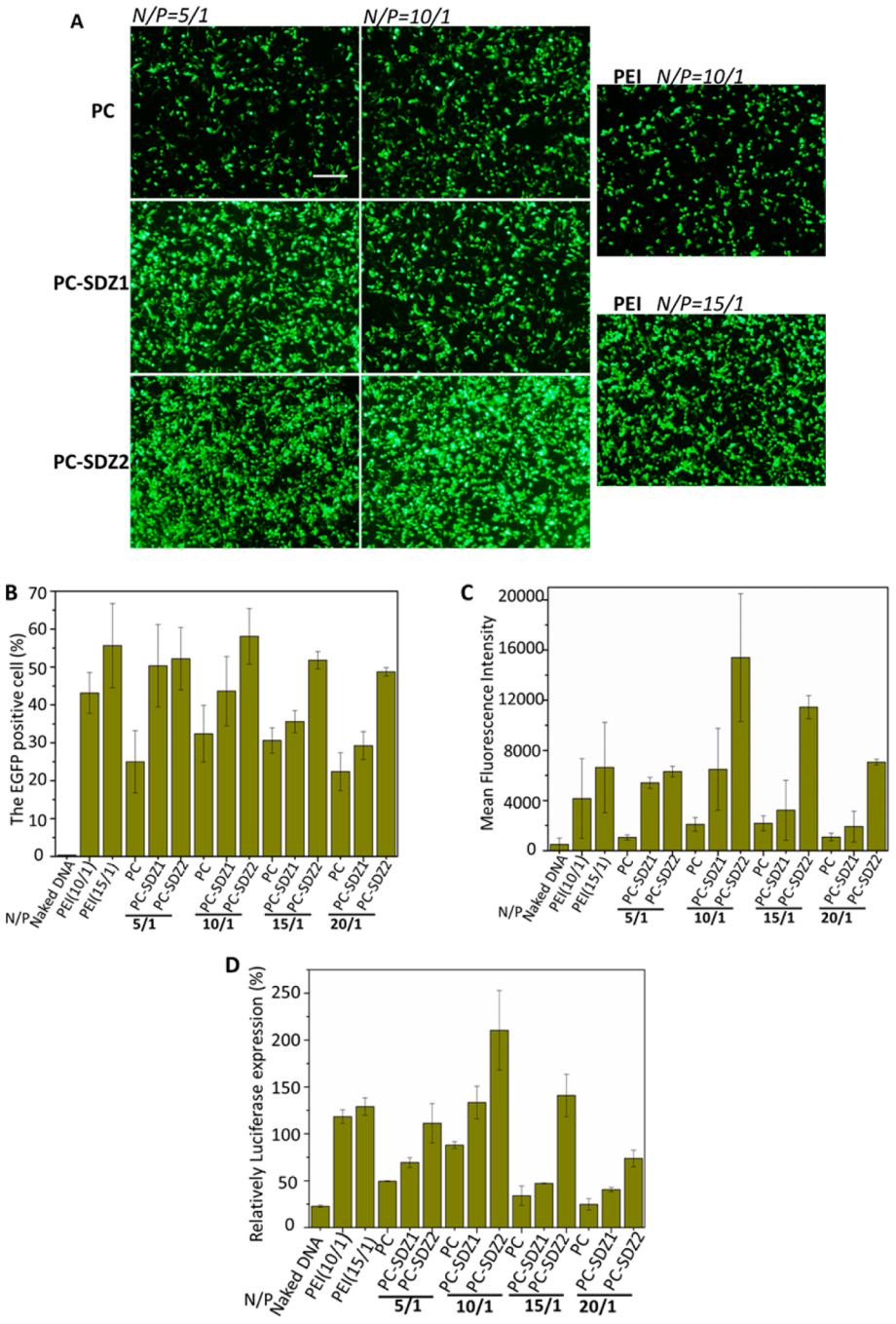

Figure 5. (A) Fluorescence images of HEK 293T cells transfected by NPs/pEGFP-N1 plasmid complexes at N/P ratios from 5 to 10. (B) Percentage of GFP positive HEK 293T cells transfected by NP/pEGFP-N1 complexes at different N/P ratios of 5 to 20. (C) Mean fluorescence intensity of GFP positive HEK 293T cells transfected by NPs/pEGFP-N1 complexes at different N/P ratios of 5 to 20. (D) In vitro transfection efficiency determined by the dual luciferase reporter gene assay of NP/pmirGLO complexes in 293T cell lines at various N/P ratios of 5 to 20. Scale bar is 100 μm. Blank cell is used as negative control; PEI (25 kDa, at its optimal N/P ratios of 10/1 and 15/1) is used as positive control.

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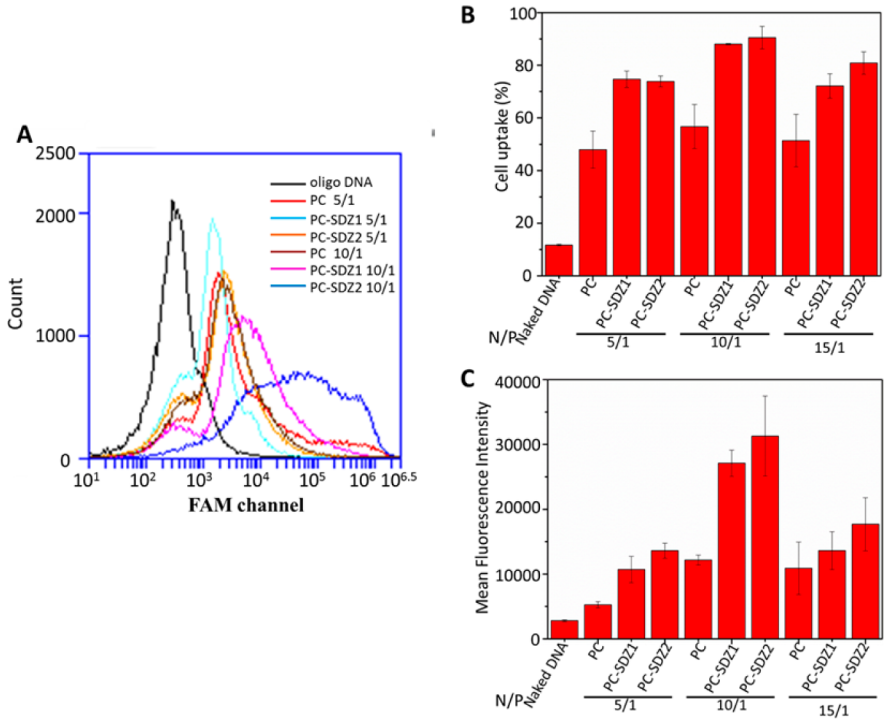

Figure 6. (A) Intracellular fluorescence intensities of NP complexes with FAM-labeled oligo-DNA determined by flow cytometry. (B) Percentage cellular uptake. The negative control was the group transfected with naked FAM-labeled oligo-DNA. (C) Mean fluorescence intensity of NP complexes with FAM-labeled oligo-DNA measured by flow cytometry.

were considered to simulate the physiological environment. As shown in Figure 4A, under pH 7.4, DNA can be loaded by PC, PC-SDZ1, and PC-SDZ2 NPs at N/P ratio of 3 and higher than 3, which means the introduction of SDZ moieties does not affect the DNA loading. As shown in Figure 4B, under pH 5.0, unloading DNA occurs in the PC NPs/DNA at the N/P ratios of 3/1 and 5/1 and in PC-SDZ1 NPs/DNA at the N/P ratio of 3/1 but not in PC-SDZ2 NPs/DNA at various N/P ratios, which indicates that the sulfadiazine modification does good to protection for gene during the course from endosome to the cytoplasm or nucleus. For PC NPs, whose shell is composed by shorter PDMAEMA chains with 15 units of DMAEMA, the shell swells because of protonation at pH 5.0 could lead to the unloading of DNA in the case of low N/P ratios, while for the PC-SDZ2 NPs, the hydrophobicity of SDZ moieties at pH 5.0 could confine the shell swelling and keep loading DNA stably. Therefore, the sulfadiazine modification can improve the stability of DNA payload in the endosome.

In Vitro Transfection. To investigate gene transfection efficiency of NPs in vitro, HEK 293T cells and MCF7 cells were transfected with NPs/pEGFP-N1 or pmirGLO complexes. Figure 5A and Figures S3 and S4, Supporting Information, show the green fluorescence images of the transfected HEK 293T cells and MCF7 cells at different N/P ratios for PC, PC-SDZ1, and PC-SDZ2 NPs and reflect the transfection capability is enhanced with the sulfadiazine portion increasing. Figure 5B shows that the percentage of GFP-positive cells transfected by PC-SDZ2 NPs/DNA complexes is 2−3-fold of the one for PC NPs/DNA complexes and its optimal N/P ratio for transfection is 10/1, at which the transfection efficiency is much better than that of commercial gold standard PEI. The same result is proved in the MCF7 cell line as shown in Figure S4, Supporting Information. Furthermore, the highest mean green fluorescence intensity for PC-SDZ2 NPs is observed at N/P ratio of 10, which is 3−4-fold and 7−8-fold compared to PEI and PC NPs, respectively, as shown in Figure 5C. For polycation gene vectors, increasing N/P ratios can help the cellular uptake due to more positive charges of complexes for the contact to the cell membrane and also enhance endosomal escape due to more proton-buffering units. However, higher N/P ratios can bond DNA stronger, which would prevent the DNA release from the complexes, which leads to transfection efficiency decrease with N/P ratios converting from 10 to 20. Therefore, optimal transfection efficiency is observed at N/P ratio of 10/1 because of the balance of cellular uptake, endosomal escape, and DNA release. Besides, the luciferase expression levels in Figure 5D further validate the transfection improvement via introduction of sulfadiazine and also show that PC-SDZ2 NPs/DNA complexes present the highest transfection efficiency, much higher than that for the gold standard PEI in HEK 293T cells.

Undoubtedly, the structural modification of sulfadiazine on polycation can significantly enhance the gene transfection efficiency. Besides the advantage afforded by SDZ to load DNA stably in the endosome environment, the effects of SDZ on the cellular uptake and intracellular distribution of NPs/DNA complexes are considered to be a major contribution to enhanced transfection efficiency which will be discussed below.

Cellular Uptake and Intracellular Distribution of NPs/DNA Complexes. FAM-labeled oligo-DNA was used to investigate the uptake capability of NPs/DNA complexes at different N/P ratios. The incorporation of sulfadiazine indeed promotes the uptake efficiency (Figure 6A,C). The percentage of the positive cell activated by PC-SDZ1 or PC-SDZ2 NPs/FAM-labeled oligo-DNA complexes reaches the highest value of more than 80% at an optimal N/P of 10 (Figure 6B). Figure 6C shows that the PC-SDZ1 and PC-SDZ2 NPs/FAM-labeled oligo-DNA complexes present the highest mean fluorescence

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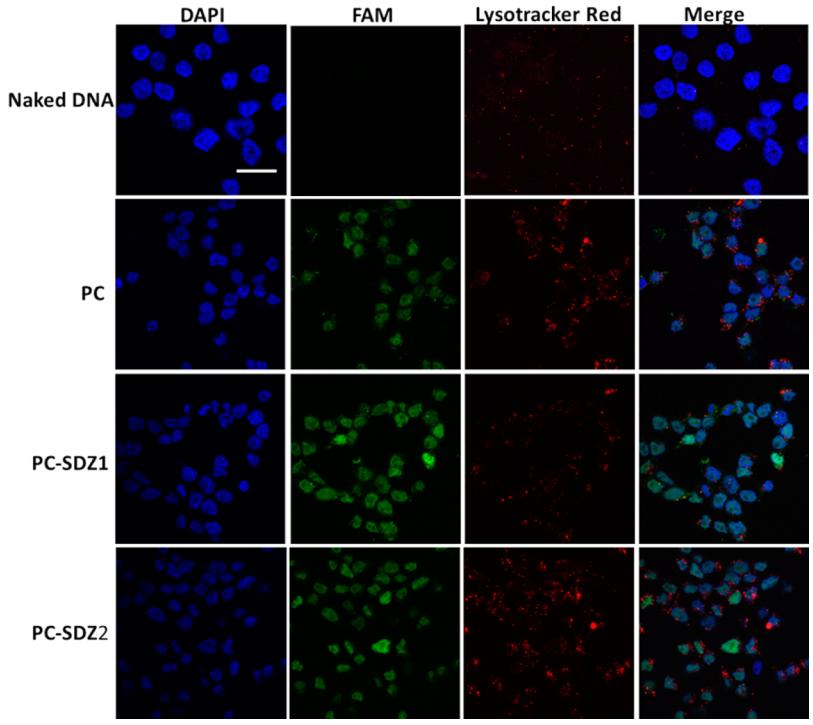

Figure 7. Intracellular distribution of transfection reagents in HEK 293T cells observed using confocal laser scanning microscopy and FAM labeled Oligo-DNA (green); late endosomes/lysosomes were stained with LysoTracker Red (red), and cell nuclei were stained with DAPI(blue). Each scale bar represents 25 μm.

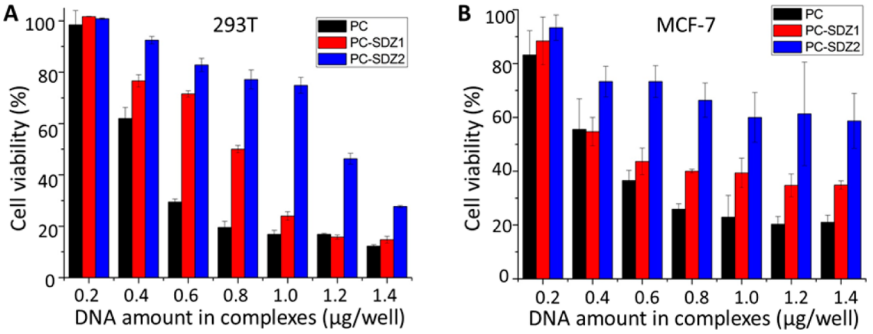

Figure 8. In vitro cytotoxicity of NP/DNA complexes in HEK 293T cells (A) and MCF-7 cells (B) following 24 h treatment evaluated by the CCK8 assay at different DNA doses for in vitro transfection. The N/P ratio of all complexes was kept at 10 which was the optimized value.

intensity at the N/P ratio of 10. At the same N/P ratio, the fluorescence intensities are strengthened with the increasing moieties of sulfadiazine, which can be explained by the improved hydrophobicity of the NPs shell facilitating the cellular uptake of carrier.

Further, the effect of sulfadiazine modification on intracellular distribution was observed by confocal microscopy. As shown in Figure 7, the FAM fluorescence is distributed on the cells uniformly, indicating that the DNA is released from the NPs complexes successfully and thoroughly, which is contributed to lower charge intensity (Figure 2B) compared to the previous study.[19] In addition, the fluorescence intensity is promoted after sulfadiazine modification and more FAM-labeled DNA is distributed on cell nucleus, which can support the EGFP protein expression being enhanced. The endosomal escape ability of polycation carriers indeed is promoted via the induction of sulfadiazine due to the proton sponge effect which was determined by the buffering capacity of polymers. Meanwhile, the pH-dependent conformational changes[40] or aqueous-solubility transition of sulfadiazine also can destabilize endosomal-membrane effectively.[27,41] This result would also be beneficial to explore the mechanism of DNA transfection which was still kept relatively limited compared to siRNA transfection.[26,42]

Cytotoxicity of NPs/DNA Complexes. Cationic copolymer/DNA complexes can lead to high toxicity in cell lines due to their positive charge on the surface of complexes. In our study, the CCK8 assay or MTT assay was performed to evaluate the in vitro toxicity of PC and PC-SDZ NPs/DNA complexes in different cell lines at N/P ratio of 10 where the highest transfection efficiency was observed above. As shown in Figure 8A,B, PC-SDZ1 and PC-SDZ2 NPs/DNA complexes show obviously higher cell viabilities than PC NPs/DNA complexes both on HEK 293T cells and MCF7 cells. In other words, with more SDZ moieties increasing, the toxicity of the polycation NPs/DNA is reduced and PC-SDZ2 NPs/DNA complexes present the highest cell biocompatibility even at high DNA doses, which could meet the different dose requirements

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especially in vivo application. Similar results also were observed in the HeLa cell line as shown in Figure S5, Supporting Information. PC, PC-SDZ1, and PC-SDZ2 NPs/DNA complexes at the N/P ratio of 8 all have relatively low cytotoxicity due to their relatively short polycation chains compared to previous reports,[43,44] and the cell viability of NPs is further promoted after being modified via sulfadiazine. The above consequences are closely related to the zeta potential as shown in Figure 2B. The shorter polycation chain and the interaction between polyanion and polycation cause the lower densities of positive charge, which makes the good biocompatibility.

CONCLUSIONS

To address the dilemma of cationic polymer nanoparticles in gene delivery with high transfection efficiency normally being accompanied with higher cell toxicity, sulfadiazine (SDZ) coordinated short-chain poly(dimethylamino ethyl methacrylate) was grafted on poly(ε-caprolactone) via ROP and ATRP. The study results indicate that the introduced sulfadiazine moieties could mediate the physicochemical properties of the NPs assembled by the amphiphilic cationic polymers especially the polycation shell's properties, including lowering the zeta potential, strengthening proton buffer capability, and enhancing hydrophobicity. With this intervention in structure and properties, in the case of short cationic chains (only 15 units), the cationic NPs/DNA complexes possess efficient DNA loading capacity, improved endosome stability, enhanced cellular uptake, the lysosomal or endosomal escape ability, and subsequently the expected higher gene transfection efficiency with lower cytotoxicity. These advantages indicate PC-SDZ can possess significant potential in vivo application. Overall, combination of SDZ and short-chain polycation is a practical and facile strategy to overcome the barrier of the efficiency and toxicity relationship of cationic nonviral gene delivery vectors.

ASSOCIATED CONTENT

Supporting Information

Figure S1, ^{1}H NMR spectroscopy of methacryloylated sulfadiazine (SDZ) in $(CD_3)_2SO$. Figure S2, ^{1}H NMR spectroscopy of P(CL-*co*-BMPCL) in $CDCl_3$. Figure S3, fluorescence images of HEK 293T cells transfected by NPs (PC, PC-SDZ1, PC-SDZ2)/pEGFP-N1 plasmid complexes at N/P ratios from 15 to 25. Figure S4, fluorescence images of MCF7 cells transfected by NPs (PC, PC-SDZ1, PC-SDZ2)/pEGFP-N1 plasmid complexes at N/P ratios of 10. Figure S5, cell viabilities of all NPs/DNA complexes at different N/P ratios of 10 against HeLa cells by MTT assay in vitro. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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