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Alkylated Sulfonium Modification of Low Molecular Weight Polyethylenimine to Form Lipopolymers as Gene Vectors

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Cite This: *ACS Omega* 2024, 9, 2339−2349

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ABSTRACT: Hydrophobic modification of low molecular weight polyethylenimine (PEI) is an efficient method to form ideal gene-transfer carriers. Sulfonium—a combination of three different functional groups, was conjugated onto PEI 1.8k at a conjugation ratio of 1:0.1 to form a series of sulfonium PEI (SPs). These SPs were hydrophobically modified and characterized by Fourier transform infrared and HNMR. DNA-condensing abilities of SPs were tested with gel retardation experiment, and their cytotoxicity was evaluated via the MTT assay. The particle size and zeta potential of SP/DNA nanoparticles were measured and evaluated for cellular uptake and transfection ability on HepG2 cell line. The results showed that the sulfonium moiety was attached to PEI 1.8k with a high yield at a conjugation ratio of 1:0.1. SPs containing longer alkyl chains condensed DNA completely at an SP/DNA weight ratio of 2:1. The formed nanoparticle size was in the range of 168−265 nm, and the zeta potential was +16−45 mV. The IC_{50} values of SPs were 6.5−43.2 μg/mL. The cytotoxicity of SPs increased as the hydrophobic chain got longer. SP/DNA showed much stronger cellular uptakes than PEI 25k; however, pure SPs presented almost no gene transfection on cells. Heparin release experiment showed that SP's strong binding of DNA resulted in low release of DNA and thus hindered the gene transfection process. By mixing SP with PEI 1.8k, the mixture presented adjustable DNA binding and releasing. The mixture formed by 67% SP and 33% PEI 1.8k showed strong gene transfection. In conclusion, sulfonium is an effective linkage to carry hydrophobic groups to adjust cell compatibilities and gene transfection capabilities of PEI.

1. INTRODUCTION

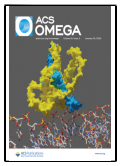
Gene therapy has become an encouraging method for treating many serious diseases, such as cancer or genetic diseases. Gene vectors are used to transfer genes into target cells in the therapy process, and nonviral gene vector stands out as promising media in recent years.[1−4] Polyethylenimine (PEI) has been one of the most extensively studied gene vectors in the past decades.[5−9] However, high molecular weight PEI has good transfection efficiency but high toxicity. On the other side, low molecular weight PEI (LMW PEI) is less toxic but has lower transfection efficiency. Modification on LMW PEI is an effective method to obtain vectors with both high transfection efficiency and low toxicity.[10,11]

Hydrophobic modification can facilitate DNA compacting via additional hydrophobic interaction and increase the lipophilicity of polymer/DNA particles which could benefit the cell membrane interaction and cellular uptake.[12−14] Through modification, the charge density of PEI is reduced, and the cytotoxicity is changed. The essential features of the hydrophobic modification on PEI could be achieved by changing the length of the hydrophobic alkyl[15−20] or aryl[21] fragments, by varying the modification degree,[22] and by switching the linker between the charged backbone and the hydrophobic groups.[23]

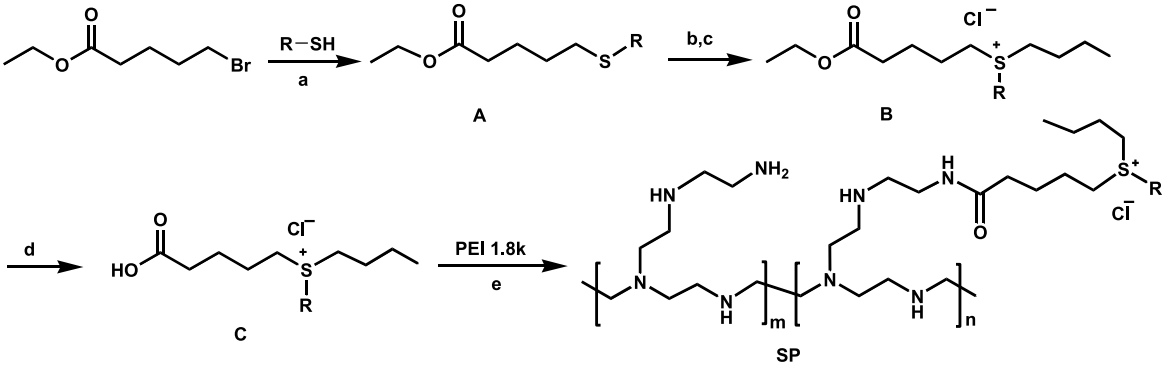
One of the most widely used methods of incorporating the hydrophobic groups onto LMW PEI is chemical grafting of the backbone amine with hydrophobic side groups by chemical reactions of quaternization, amidation, alkylation, acylation, or Schiff-base reduction.[14] Biologically degradable linkages are preferable in the synthesis, such as ester, amide, acetal, carbamate, or urea.[14] These existing studies aimed to explore the structure−function relationship of the hydrophobic group and the degree of substitution. Yet, more detailed works are waiting for rational design in the functional groups for PEI modifications.

Various functional groups were attached onto LMW PEI to achieve a better gene vector. Neamnark[24] reported the aliphatic lipid substitution of PEI 2000 by coupling various linear aliphatic acids with the PEI amine to find the lipid influence on transfection. Teo[25] linked the alkyl group (ethyl octyl, dodecyl)/aromatic function group, benzyl, or urea to hydrophobic modified PEI 1.8k using a methyl-carboxytrimethylene carbonate monomer to study the influences of the functional group and the substitution degree on the transfection efficiency. Shen[26] grafted hydrophobic ligands including alkane, cycloalkanes, and fluoroalkanes onto PEI to study the siRNA delivery and found that fluoroalkylated PEIs showed higher efficacies. Yu[27] reported the aromatic modification of PEI 1.8k by incorporating tryptophan and phenylalanine onto PEI 1.8k and noted that tryptophan can intercalate into DNA and enhance the condensation. Yang[28]

Received: August 23, 2023
Revised: December 14, 2023
Accepted: December 18, 2023
Published: January 4, 2024



Scheme 1. Synthetic Scheme of the Targets[a]



Compound:	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4	SP1	SP2	SP3	SP4
R:	C_3H_7	C_8H_{17}	$C_{14}H_{29}$	$C_{16}H_{33}$	C_3H_7	C_8H_{17}	$C_{14}H_{29}$	$C_{16}H_{33}$	C_3H_7	C_8H_{17}	$C_{14}H_{29}$	$C_{16}H_{33}$	C_3H_7	C_8H_{17}	$C_{14}H_{29}$	$C_{16}H_{33}$
yield (%):	69	74	72	74	64	63	69	46	39	49	53	48	96	80	77	45

[a](a) NaOH, DMF, rt, 24 h; (b) *n*-BuI, $AgBF_4$, CH_3CN, 68 °C, 24 h; (c) resin Cl^-, 2 h; (d) NaOH 10%, EtOH, 50 °C, 1 h; and (e) EDC, NHS, DMF, rt, 48 h.

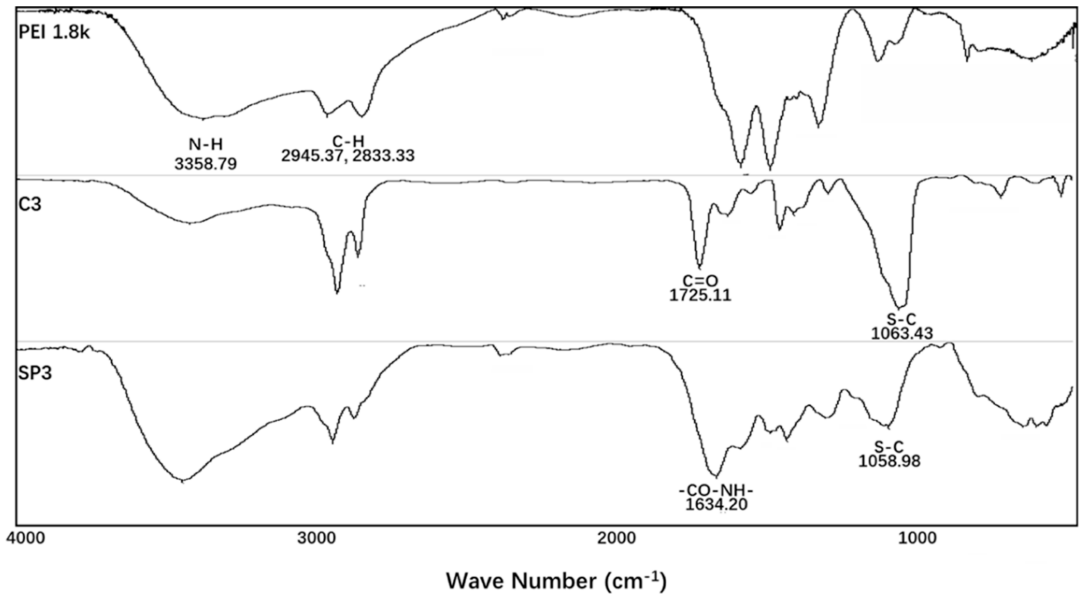


Figure 1. FT-IR spectra of PEI 1.8k, C3, and SP3.

conjugated PEI with a phospholipid that contains two aliphatic chains on the glycerol skeleton. Other hydrophobic modifications such as folate[29] and cholesterol[30,31] have been investigated for the benefits of their targeting specificities.

Sulfur-containing compounds have been widely studied for their vast application. Introduction of a sulfur moiety into a molecule can dramatically change its biological activity by modifying atom and binding parameters. Sulfonium, containing a constant positive charge and three branches, has been studied in antibiotic,[32−35] antidiabetes,[36] enzyme inhibitors,[37] and anticancer drugs.[38] Sulfonium was also reported to have gene delivery ability.[39,40] In some aspects, sulfonium has been proven to have many features similar to quaternary ammonium[41] and can be used as an isostere for a positively charged nitrogen center.[42]

In the matter of synthesis, the substitute groups of amine or quaternary ammonium are less controllable; the substitution variability is restricted by the difficulty of selective synthesis of secondary, tertiary amine, or quaternary ammonium, while in the formation of the C−S bond, it is more stepwisely controllable. The composition of the substitute groups of sulfur could be selectively designed and synthesized step by step with a strict structure. Innovation of a new methodology for the synthesis of thioether and sulfonium, such as catalytic reaction,[43] water-promoted formation,[44] and "click" type reaction,[45] is also under exploration for an economical and greener strategy. These provide practical methods of building functional groups around the sulfur center to achieve the expected purpose. Methylated sulfonium was reported several times in the formation of polymers.[39,45] However, the design of sulfonium structure is still lacking diversity, and the knowledge of sulfonium-mediated gene transfection is limited.

In this study, we aimed to explore the possibility of using sulfonium as the linkage to graft alkyl chains onto PEI 1.8k to adjust the lipophilicity and to find the balance between charge density and lipophilicity. The sulfonium center was composed

https://doi.org/10.1021/acsomega.3c06255
ACS Omega 2024, 9, 2339−2349

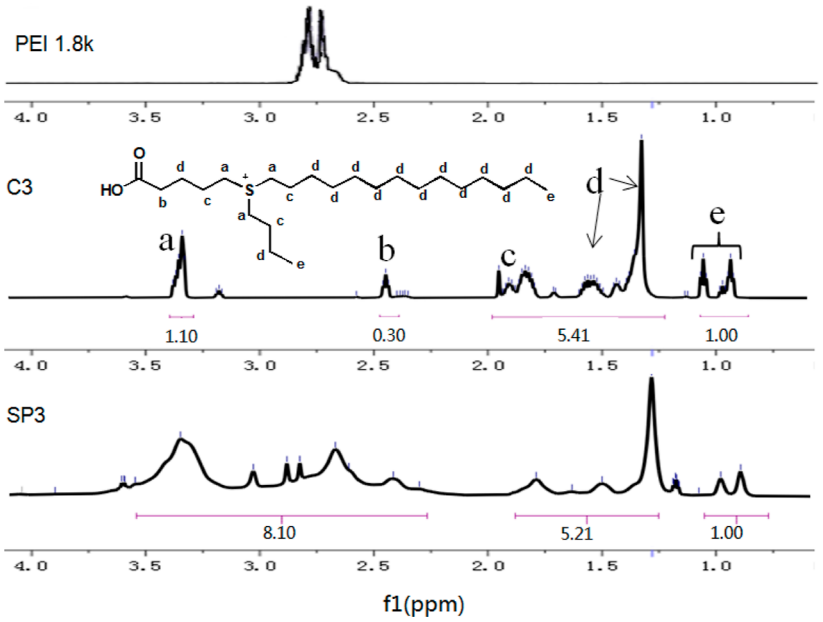


Figure 2. ^{1}H NMR spectra of PEI 1.8k, C3, and SP3.

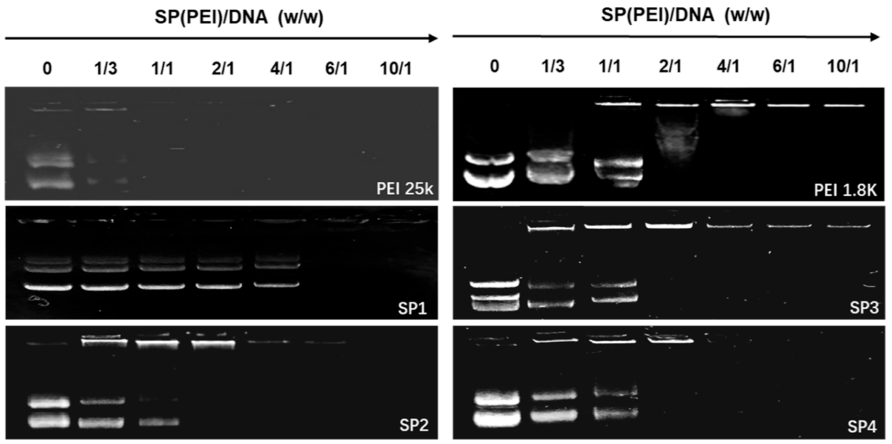


Figure 3. Agarose gel electrophoresis images of SP (PEI)/DNA condensed at different mass ratios (w/w). 0 is naked DNA; 1/3, 1/1, 2/1, 4/1, 6/1, and 10/1 represent the mass ratio of SP (PEI) to DNA, respectively.

of two alkyl chains and one carboxylic acid. The conjugation was performed by coupling the carboxylic acid group with the amine of PEI by using carbodiimide chemistry. The formed sulfonium PEIs (SPs) were investigated for gene delivery ability.

2. RESULTS

2.1. Synthesis of SPs. The synthesis of SPs is summarized in Scheme 1. In general, 5-bromopentanoate reacted with alkyl mercaptan to form thioether **A** that was treated with butyl iodide to construct sulfonium moiety **B** which was then hydrolyzed to form sulfonium acid **C**. SPs were prepared by amide conjugation of the amines of PEI 1.8k and the carboxylic acid of sulfonium compound **C** at a mole ratio of 1:0.1. The mole of PEI was calculated using $CH_2CH_2NH_2$ as a unit. The amide formation was carried out using active agent EDC/NHS. After dialysis and lyophilization, SPs were obtained as yellowish gel/foam products. Compounds **B** and **C** were characterized by mass, ^{1}HNMR, and ^{13}CNMR. Their spectra were attached in Supporting Information Figures S1−S8.

2.2. Characterization of SPs. The successful synthesis of SPs was proved by the characterization of initial materials and products by IR and ^{1}HNMR spectroscopy. The IR and ^{1}HNMR spectra of SP1, SP2, and SP4 are provided in Supporting Information Figures S9 and S10. PEI 1.8k, C3, and SP3 were characterized, and the spectra are shown in Figures 1 and 2. In the PEI spectrum, 3358.79 cm^{-1} is the N−H vibration. In the IR spectrum of **C3**, the peak at 1725.11 cm^{-1} is the stretching vibration of C═O, and the peak at 1063.43 cm^{-1} is the vibration of S−C. In the spectra of SP3, a new amide peak appears at 1634.20 cm^{-1}, and the S−C peak appears at 1058.98 cm^{-1}.

Figure 2 presents the ^{1}H NMR spectra of PEI 1.8k, C3, and SP3. Protons of **C3** are assigned as labeled. Peaks in the range of 3.37−3.32 ppm belong to protons **a** which are next to sulfonium directly; peaks at 2.44 ppm are protons **b** that are triplet obviously split by two neighboring protons. Peaks from 1.91 to 1.76 ppm are overlaps of protons of **c** which are secondary adjacent to sulfonium; peaks from 1.58 to 1.30 ppm are overlaps of protons of **d**. Two peaks at 1.03 and 0.91 ppm

https://doi.org/10.1021/acsomega.3c06255
ACS Omega 2024, 9, 2339−2349

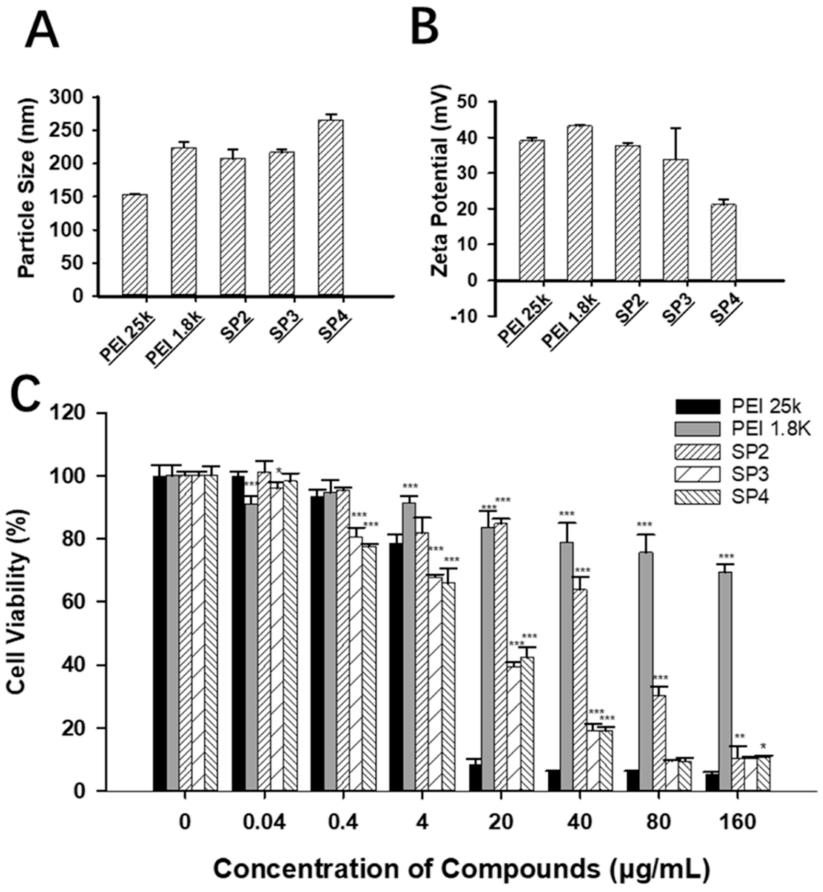


Figure 4. (A,B) Particle size and zeta potential of SP(PEI)/DNA condensates formed by adding aqueous GFP plasmid solution (28 μL, 800 ng) to solutions (28 μL) of SP(PEI) in HEPE (pH = 7.5, 5 mmol/L) at a weight ratio of 2/1 (mean ± SD, n = 3). (C) Cell viability of PEI 25k, PEI 1.8k, and SPs on HepG2 cells at 48 h determined by the MTT assay (mean ± SD, n = 5). *Significant difference compared to the positive control, PEI 25k, of the same concentration ($p < 0.05$); **very significant difference compared to the positive control, PEI 25k, of the same concentration ($p < 0.01$); and ***extremely significant difference compared to the positive control, PEI 25k, of the same concentration ($p < 0.001$).

are chemical shifts of protons **e** of two CH_3. In SP3, proton signals are almost the combination of PEI 1.8k and C3. The actual modification ratios were confirmed by comparing the integrations of PEI peaks from 3.54 to 2.47 ppm and the integrations of CH_3 at 1.10 and 0.95. Upon calculation, about 3.9 molecules of C3 were conjugated onto PEI 1.8k in SP3. Similar amounts of sulfonium moieties were attached in SP1, SP2, and SP4 according to the spectra in Figure S1.

2.3. Gel Retardation of SP/DNA. The DNA binding capacity of SP was evaluated using the gel retardation assay at various weight ratios of 1/3, 1/1, 2/1, 4/1, 6/1, and 10/1. The results are shown in Figure 3. PEI 1.8k presents retardation at a ratio of 6/1, and PEI 25k presents retardation at a ratio of 1/1. SP1 was retarded at 6/1. SP2, SP3, and SP4 showed retardation of DNA at a weight ratio of 2/1. SP2, SP3, and SP4 presented stronger binding capacity than PEI 1.8k. The hydrophobic interactions of alkyl chains in the complexes facilitate the stable assembly of the nanoparticle.

2.4. Particle Size and Zeta Potential of SP/DNA Condensate. DNA condensates of SP2, SP3, and SP4 were measured in the particle size and zeta potential at a weight ratio of 2/1. The results are shown in Figure 4A,B. The SP/DNA particle size is in the range of 168−265 nm, and the zeta potential is in range of +16 to + 45 mV, which are similar to those of PEI 25k/DNA and PEI 1.8k/DNA.

2.5. Cytotoxicity of SPs. The cell toxicities of SP2, SP3, and SP4 were evaluated via the MTT assay on HepG2 cells at 48 h. PEI 25k and PEI 1.8k were tested for comparison. The results are shown in Figure 4C. The cytotoxicities of these polycations are dose dependent, and their IC_{50} values are 6.5−43.2 μg/mL. SP2, SP3, and SP4 become more toxic than PEI 1.8k but less than PEI 25k. SPs with a longer alkyl chain present higher toxicity than that with a shorter chain.

2.6. Cellular Uptakes and Gene Transfection of SP/DNA Condensate. Cellular uptakes of SP(PEI)/Cy5-eGFP were carried out on HepG2 cells at a weight ratio of 2/1 without serum. The results were observed under a Leica TCS SP8 and are shown in Figure 5A. The cellular uptake of SP2/DNA was very low, while SP3 and SP4 showed strong uptake. The fluorescence intensities were quantitatively analyzed using image pro plus 6, as shown in Figure 5B; SP3 and SP4 were 4 and 1.7 times stronger than PEI 25k, respectively. SP3 and SP4 were then evaluated for gene transfection ability using eGFP as the green fluorescent protein reporter gene, but both SP3 and SP4 presented a much weaker green fluorescence as shown in Figure 5C.

2.7. DNA Release of SP/DNA Condensate by Heparin. SP3(SP4)/DNA particles were tested for DNA release at different concentrations of Heparin. PEI 25k and PEI 1.8k were set for comparison. As shown in Figure 6A, PEI 25k/DNA released DNA at a heparin concentration of 2 U/μg

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ACS Omega 2024, 9, 2339−2349

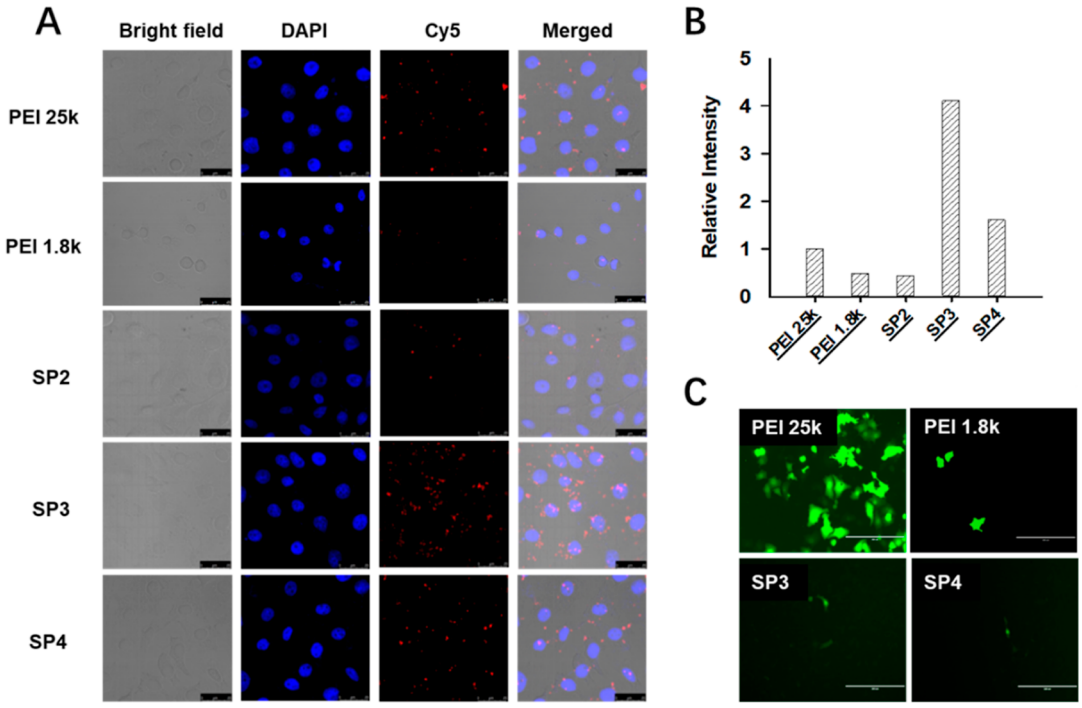

Figure 5. (A) Determination of intracellular distribution of SP(PEI)/Cy5-eGFP condensation products at 4 h under serum-free conditions at a weight ratio of 2:1. Images obtained under a transcription microscope. eGFP was labeled through Cy5 (red); the nuclei were dyed by DAPI (blue); scales are 25 μm. (B) Colocalization ratio of the complexes containing Cy5-eGFP with nuclei, calculated using software image pro plus 6.0. (C) Determination of transfection ability of SP(PEI)/eGFP condensation production of HepG2 cells under serum-free conditions. SP(PEI)/DNA, w/w = 2/1, scales are 200 μm.

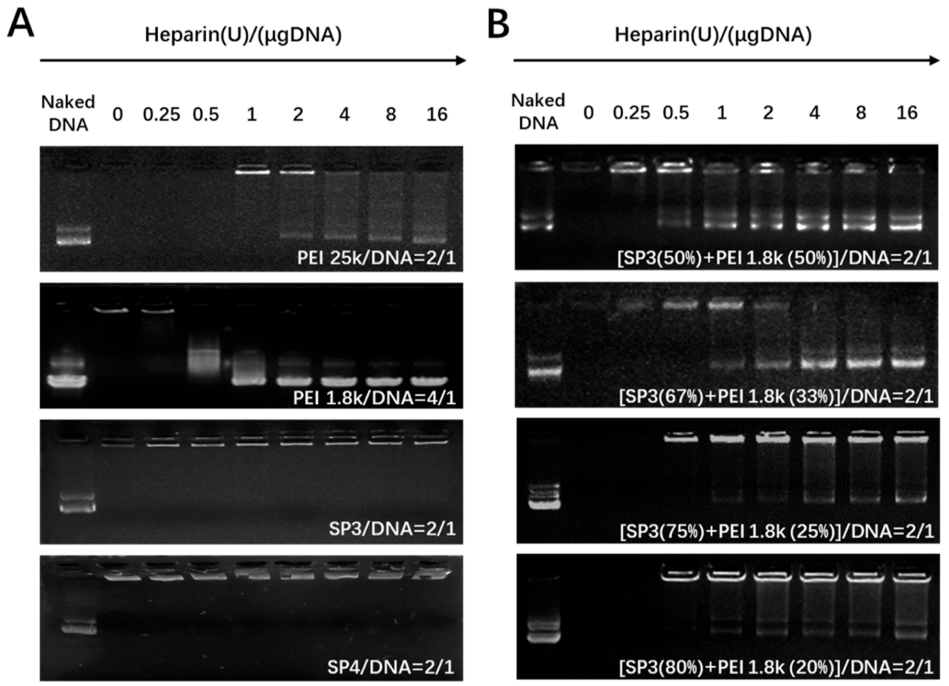

Figure 6. (A) Agarose gel electrophoresis images of heparin release of SP(PEI)/DNA. PEI 25k, SP3, and SP4 were tested at a mass ratio of 2/1, and PEI 1.8k was tested at a ratio of 4/1. The heparin concentrations were of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 U/μg DNA. (B) Agarose gel electrophoresis images of heparin release of DNA condensates formed by SP3 and PEI 1.8k mixture at various percentages. The heparin concentrations were of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 U/μg DNA.

DNA, and PEI 1.8k/DNA released DNA at a heparin concentration of 0.5 U/μg DNA and completely released at 1 U/μg DNA. SP3 and SP4 did not release DNA at a heparin concentration of up to 16 U/μg DNA.

To adjust the binding effect of SPs, SP3 was selected to mix with PEI 1.8k at weight percentages of 50, 33, 25, and 20%. These mixtures were condensed with DNA to form particles and then were tested for DNA release level in heparin. The

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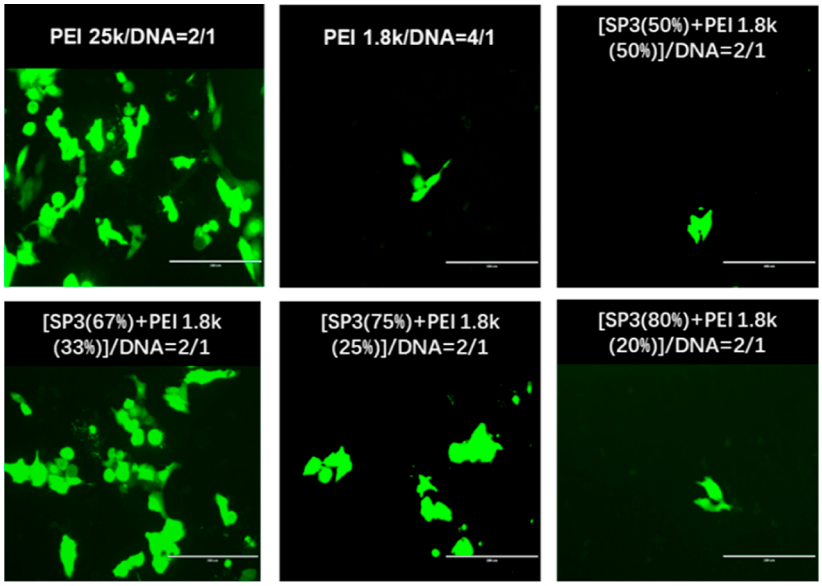

Figure 7. Determination of transfection ability of (SP3+PEI1.8k)/eGFP condensation production of HepG2 cells under serum-free conditions. Scales are 200 μm.

results are shown in Figure 6B. First of all, the mixtures presented effective DNA condensation at a weight ratio of 2:1. When treated with heparin, the mixture containing 50% PEI 1.8k released DNA at a heparin concentration of 0.5 U/μg DNA. When the mixture contained 33% PEI 1.8k, DNA was released at a heparin concentration of 1 U/μg DNA. At 25 and 20% PEI 1.8k mixing, less DNA release was observed.

2.8. Gene Transfection of (SP + PEI 1.8k)/DNA Condensate. The mixtures of SP3 and PEI 1.8k were condensed with the eGFP plasmid at a weight ratio of 2:1 to form nanoparticles and were evaluated for the gene transfection effect on HepG2 cells. As shown in Figure 7, a mixture of SP3 (67%) and PEI 1.8k (33%) gave strong fluorescence. The mixtures with other ratios were less effective.

3. DISCUSSION

Ammonium has been used widely in the development of the nonviral gene vector. Heteroatoms such as phosphonium and sulfonium have been gradually investigated in gene delivery ability recently. Sulfonium has a constant positive charge, making it capable of playing a similar role as quaternary ammonium to interact with negatively charged DNA.[39] The advantages of sulfonium lie in the easy synthesis and selective attachment of three functional moieties.

To systematically study the functions of sulfonium, we started with simple sulfonium composed of three arms: one is a carboxylic acid group which would react with amine, one is an alkyl chain with a fixed length of 4 carbon, and another aliphatic chain varied from 3 carbon to 16 carbon to perform a hydrophobic effect. These sulfonium compounds were grafted onto PEI 1.8k via amide condensation. Upon conjugation, SPs were obtained, and the total positive charge of PEI was maintained, which could retain the electrostatic force with negative DNA. The linear alkyl chains enhanced the hydrophobic interaction in the formation of SP/DNA particles.

The conjugation of PEI 1.8k and sulfonium was preceded at a weight ratio of 1:0.1. The successful conjugation was proved by the formation of an amide bond at 1634.20 cm^{-1} in Fourier transform infrared (FT-IR) spectra. The conversion rate was determined by the ^{1}H NMR spectra. In PEI 1.8k, there are about 42 units of $CH_2CH_2NH_2$, and 4.2 molecules of sulfonium **C** were supposed to be conjugated onto PEI at a ratio of 1:0.1. ^{1}H NMR spectra of SP1-SP4 indicated that about 3.85−4.10 molecules of sulfonium were attached onto PEI 1.8k, which means that the sulfonium moieties were attached onto PEI 1.8k at high yield.

The DNA condensing abilities of SPs were verified by gel retardation. From the experiments, the alkyl chain length of the sulfonium moiety influences the polymer's DNA binding capacity. When the alkyl chain is short (SP1), the condensation is similar to PEI 1.8k. When the alkyl chain gets longer, the polymers (SP2, SP3, and SP4) present increasing DNA binding ability. Their condensations appear at a weight ratio of 2:1, which is better than PEI of 1.8k at 6:1.

Based on the electrophoresis, the DNA condensates of SP2, SP3, and SP4 were measured in particle size and zeta potential at a weight ratio of 2:1. All of the particles have size in the range of 168−265 nm, and the zeta potentials are between +16 and 45 mV. These particles are suitable for cellular uptake.

In the cytotoxicity experiment, the polymers' IC_{50} values are 6.5−43.2 μg/mL on HepG2 cells. They are less toxic than PEI 25k but more toxic than PEI 1.8k. SP2 containing shorter lipids is less toxic, while SP3 and SP4 containing longer lipids get more toxic. Studies of PEI showed that primary and secondary amines increase toxicity, while tertiary amines reduce toxicity.[46,47] The toxicity of sulfonium is reported comparable to quaternary amine.[40] The primary amine was replaced with tertiary sulfonium, which should have a positive effect on cell viability. The lipid modification and the linear extension of the sulfonium moiety aggravate the disturbance to the cell membrane and cause cell lyses. The overall effect of the modification on PEI of 1.8k leads to increased cytotoxicity.

Cellular uptake images were obtained at an SP(PEI)/DNA weight ratio of 2:1 and at a final SP(PEI) concentration of 3 μg/mL, which is lower than the IC_{50} value, in serum-free condition. From the results, SP3/DNA and SP4/DNA present 4 times and 1.7 times stronger cell penetration than PEI 25k, respectively. However, in gene transfection experiment, SP3

https://doi.org/10.1021/acsomega.3c06255
ACS Omega 2024, 9, 2339−2349

and SP4 had very low transfection of eGFP. We examined the binding stability using the DNA release experiment by heparin. The results showed that SP3 and SP4 condensed DNA very tight, and no releases of DNA occurred at high concentration of heparin.

Successful cellular uptake and DNA release are two of the major steps in the gene transfection pathway. The balance between the charge density and the hydrophobic interaction is important in the discovery of a gene vector. In previous reports, the hydrophobic modification degrees have varied largely. In Neamnark paper,[24] 0.2−6.9 chain lipids were conjugated onto PEI 2.0k. Teo's products[25] had 1−10 substitutions per PEI 1.8k. Yu used 9−22 aromatic substitutions per PEI 1.8k.[27] In these reports, the substitutions were different; the best transfection was achieved at different modification degrees.

Apparently, the modification of SPs is not effective enough to overcome these transfection barriers. The positive and negative charge ratios of SP3/DNA and SP4/DNA are 8.3 and 8.0, respectively, and the N/P ratio of PEI 25k/DNA is 15.2. The lower charge ratios of SPs potentially contributed to the less ideal results, and two factors may contribute to the strong binding: the permanent charges of sulfonium have strong interaction with phosphate of DNA, and hydrophobic interaction plays an important role in the condensation process. In SP3 and SP4, we supposed that dilution of the permanent charge and hydrophobic interaction may reduce the DNA binding ability. SP3 was then mixed with PEI 1.8k at a series of weight percentages. The mixtures were tested for DNA release, cell tolerance, and gene transfection. The results showed that the addition of PEI 1.8k to SP3 could adjust the DNA release. At the same time, the mixture was less toxic than pure SP3 (MTT results in Figure S11), and the mixture of SP3 and PEI 1.8k at ratio of 67%:33% presented much stronger gene transfection. This experiment showed the importance of the balancing of binding and release of DNA.

4. MATERIALS AND METHODS

4.1. Materials. All chemical reagents were purchased from commercial sources. Absolute dichloromethane, acetonitrile, acetone, and triethylamine were distilled after being dried with calcium hydride. All aqueous solutions were prepared with deionized water. The Enhanced Green Fluorescent Protein (eGFP) encoding plasmid DNA (pDNA) was purchased from Aldevron and cloned in *Escherichia coli*-DH5α (TIANGEN) and then extracted with the QIAGEN Mega Plasmid kit (Qiagen EndoFree Plasmid, Sigma-Aldrich). Label IT Cy5 Labeling Kit was purchased from Mirus Bio Company and was used to label eGFP to give Cy5-eGFP. The HepG2 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and stored in lab. Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Solarbio Company.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Ascend 600 M AVANCE III HD. Mass spectra (ESI) were acquired on a MASS 6540 UHD Q-TOF. Plasmid concentration was measured with a Nanodrop (Thermo Scientific 2000C). Electrophoresis gel experiments were operated on a submarine system of BIO-RAD Power PacTM Universal Power Supply. Hydrodynamic diameters and zeta potentials were determined using a Nano-ZS 90 Laser Particle Size and Zeta Potential Analyzer. The MTT assay was performed on a Thermo Scientific Multiskan GO. The fluorescence was observed using Leica biological microscope TCS SP8 or DMI 4000B.

4.2. Synthesis of SPs. General procedure for compound **A**: alkyl mercaptan (1.2 equiv) in DMF was added to saturated NaOH solution and stirred for 1 h. The mixture was then added to ethyl 5-bromopentanoate (1 equiv) and stirred overnight. The reaction was quenched with water and extracted with ether (10 mL × 3). The ether layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified with chromatography (ethyl acetated/hexane) to give compound **A**.

General procedure for compound **B**: compound **A** was dissolved in dry acetonitrile (15 mL) in a round-bottom flask, to which was then added butyl iodide (1.5 equiv). The flask was covered with aluminum foil and then was added $AgBF_4$ (1.5 equiv). The reaction was refluxed at 85 °C for 24 h and monitored by TLC. After the reaction was completed, the mixture was cooled and filtered. The filtrate was treated with ion-exchange resin (Cl^-) for 2 h and then filtered and concentrated, and the resulting residue was purified by chromatography ($MeOH/CH_2Cl_2$) to give sulfonium ester **B**.

*General Procedure for Compound **C***. Ester **B** was treated with 10% NaOH in ethanol and heated for 1 h at 50 °C. The reaction mixture was neutralized with HCl (1.1 equiv) concentration and concentrated. The residue was purified by chromatography ($MeOH/CH_2Cl_2$) to give sulfonium acid **C**.

*General Procedure for Compound **SP***. Compound **C** in dry DMF was added EDC·HCl and NHS and then stirred for 2 h. The mixture was added slowly to PEI 1.8k solution in dry DMF and stirred for 48 h at room temperature. After reaction, the mixture was dialyzed in 50% ethanol (4 h × 6) with a MWCO of 1000 Da. After dialysis, ethanol was evaporated off from the solution and then freeze-dried to give **SP** as a white gel or foam.

*S-(4-Ethoxycarbonyl-butyl)-butyl-propyl Sulfonium Chloride (**B1**)*. **A1** (0.17 g, 0.83 mmol), butyl iodide (0.14 mL, 1.25 mmol, 1.5 equiv), and $AgBF_4$ (0.24 g, 1.25 mmol, 1.5 equiv) to give product **B1** as a yellowish oil (0.14 g, 0.54 mmol, yield 64%); Rf = 0.6 ($MeOH-CH_2Cl_2$ = 1:19). ^{1}H NMR($CDCl_3$, δ) 4.13 (q, J = 6.6 Hz, 2H), 3.27−3.18 (m, 6H), 2.37 (t, J = 7.7 Hz, 2H), 1.75−1.57 (m, 8H), 1.40 (dt, J = 14.0, 7.0 Hz, 2H), 1.24 (t, J = 6.6 Hz, 3H), 0.98 (t, J = 8.2 Hz, 3H), 0.92 (t, J = 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$, δ): 173.72, 60.72, 39.37, 36.38, 36.03, 33.98, 28.37, 25.13, 21.58, 20.95, 19.02, 14.19, 13.96, 12.68; HR-MS(ESI)m/z: Calcd for $C_{14}H_{29}O_2S\{[M-Cl^-]\}$, 261.1883; found, 261.1998.

*S-(4-Ethoxycarbonyl-butyl)-butyl-octyl Sulfonium Chloride (**B2**)*. **A2** (1.24 g, 4.5 mmol), butyl iodide (0.77 mL, 6.8 mmol, 1.5 equiv), and $AgBF_4$ (1.31g, 6.8 mmol, 1.5 equiv) to give product **B2** as a yellowish oil (0.94 g, 2.84 mmol, yield 63%); R_f = 0.6 ($MeOH-CH_2Cl_2$ = 1:19). ^{1}H NMR($CDCl_3$, δ): δ 4.12 (q, J = 7.2 Hz, 2H), 3.27−3.18 (m, 4H), 3.21−3.16 (m, 2H), 2.37 (t, J = 7.6 Hz, 2H), 1.76−1.57 (m, 8H), 1.45−1.36 (m, 4H), 1.40−1.21 (m, 11H), 0.92 (t, J = 7.1 Hz, 3H), 0.89 (t, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, δ): 173.72, 60.72, 36.52, 36.29, 36.04, 33.98, 31.78, 29.33, 29.32, 28.37, 25.78, 25.64, 25.13, 22.71, 21.58, 19.02, 14.19, 14.09, 13.96; HR-MS(ESI)m/z: Calcd for $C_{19}H_{39}O_2S\{[M-Cl^-]\}$, 331.2665; found, 331.2789.

*S-(4-Ethoxycarbonyl-butyl)-butyl-tetradecyl Sulfonium Chloride (**B3**)*. **A3** (0.67 g, 1.87 mmol), butyl iodide (0.32 mL, 2.81 mmol, 1.5 equiv), and $AgBF_4$ (0.55 g, 2.81 mmol, 1.5

https://doi.org/10.1021/acsomega.3c06255
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equiv) to give product **B3** as a yellowish oil (0.50 g, 1.29 mmol, yield 69%); R_f = 0.5 ($MeOH-CH_2Cl_2$ = 1:19). ^1H NMR($CDCl_3$, δ): 4.12 (q, J = 7.1 Hz, 2H), 3.45−3.30 (m, 6H), 2.41 (t, J = 6.8 Hz, 2H),1.94−1.75 (m, 8H), 1.55−1.45 (m, 4H), 1.36−1.25 (m, 23H), 1.00 (t, J = 7.3 Hz, 3H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR($CDCl_3$, δ): 172.91, 60.62, 40.16, 40.04, 39.87, 32.90, 31.92, 29.88, 29.69, 29.65, 29.61, 29.49, 29.36, 29.28, 28.94, 28.41, 26.64, 24.81, 24.04, 23.26, 22.69, 21.68, 14.20, 14.12, 13.34; HR-MS(ESI)m/z: Calcd for $C_{25}H_{51}O_2S\{[M-Cl^-]\}$, 415.3604; found, 415.3563.

*S-(4-Ethoxycarbonyl-butyl)-butyl-hexadecyl Sulfonium Chloride (**B4**).* **A4** (0.72 g, 1.87 mmol), butyl iodide (0.32 mL, 2.81 mmol, 1.5 equiv), and $AgBF_4$ (0.55 g, 2.81 mmol, 1.5 equiv) to give product **B4** as a yellowish oil (0.38 g, 0.86 mmol, yield 46%); R_f = 0.5 ($MeOH-CH_2Cl_2$ = 1:19). ^1H NMR($CDCl_3$, δ): 4.12 (q, J = 7.1 Hz, 2H), 3.44−3.33 (m, 6H), 2.41 (t, J = 6.8 Hz, 2H), 1.92−1.76 (m, 8H), 1.55−1.45 (m, 4H), 1.37−1.25 (m, 27H), 1.00 (t, J = 7.3 Hz, 3H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR($CDCl_3$, δ): 172.90, 60.61, 40.15, 40.04, 39.91, 39.87, 32.90, 31.93, 29.70, 29.67, 29.61, 29.50, 29.37, 29.29, 28.94, 28.41, 26.63, 24.80, 24.04, 23.98, 23.26, 22.69, 21.68, 14.23, 14.20, 14.12, 13.33; HR-MS(ESI) m/z: Calcd for $C_{27}H_{55}O_2S\{[M-Cl^-]\}$, 443.3917; found, 443.4059.

*S-(4-Carboxylbutyl)-butyl-propyl Sulfonium Chloride (**C1**).* Compound **B1** (0.76 g) was applied to basic hydrolysis to give **C1** (0.28 g, 1.19 mmol) as a yellowish oil, yield 39%, R_f = 0.5($MeOH-CH_2Cl_2$ = 1:9). ^1H NMR (CD_3OD, δ): 3.34 (m, 4H), 3.20 (d, J = 6.6 Hz, 2H), 2.43 (t, J = 7.3 Hz, 2H) 1.74−1.55 (m, 8H), 1.42 (dt, J = 13.9, 6.9 Hz, 2H), 0.98 (t, J = 8.2 Hz, 3H), 0.92 (t, J = 6.7 Hz, 3H); ^{13}C NMR (CD_3OD, δ): 175.33, 39.37, 36.38, 36.03, 34.07, 28.37, 25.27, 21.78, 20.95, 19.02, 13.96, 12.68; HR-MS(ESI)m/z: Calcd for $C_{12}H_{25}O_2S\{[M-Cl^-]\}$, 233.1570; found, 233.1656.

*S-(4-Carboxylbutyl)-butyl-octyl Sulfonium Chloride (**C2**).* Compound **B2** (0.46 g) was applied to basic hydrolysis to give **C2** (0.21 g, 0.68 mmol) as a yellowish oil, yield 49%, R_f = 0.4($MeOH-CH_2Cl_2$ = 1:9). ^1H NMR (CD_3OD, δ): 3.34 (dt, J = 14.9, 6.6 Hz, 6H), 2.43 (q, J = 5.1, 2.9 Hz, 2H), 1.85 (ddq, J = 41.8, 16.1, 8.0 Hz, 8H), 1.54 (dp, J = 23.8, 7.8 Hz, 4H), 1.42−1.34 (m, 8H), 1.03 (t, J = 7.4 Hz, 3H), 0.92 (t, J = 6.9 Hz, 3H); ^{13}C NMR (CD_3OD, δ): 175.21, 38.94, 38.69, 32.26, 31.45, 28.92, 28.71, 28.54, 28.10, 25.74, 23.82, 23.25, 23.14, 22.25, 21.33, 12.99, 12.25; HR-MS(ESI)m/z: Calcd for $C_{17}H_{35}O_2S\{[M-Cl^-]\}$, 303.2352; found, 303.2466.

*S-(4-Carboxylbutyl)-butyl-tetradecyl Sulfonium Chloride (**C3**).* Compound **B3** (0.73 g) was applied to basic hydrolysis to give **C3** (0.36 g, 0.93 mmol) as a yellowish oil, yield 52%, R_f = 0.4($MeOH-CH_2Cl_2$ = 1:9). ^1H NMR (CD_3OD, δ): 3.34 (dt, J = 15.5, 6.8 Hz, 4H), 3.16 (t, J = 7.4 Hz, 2H), 2.43 (t, J = 7.2 Hz, 2H), 1.95−1.76 (m, 8H), 1.58−1.47 (m, 4H), 1.43−1.30 (m, 20H), 1.03 (t, J = 7.5 Hz, 3H), 0.91 (t, J = 7.3 Hz, 3H); ^{13}C NMR (CD_3OD, δ): 175.18, 38.95, 38.70, 32.27, 31.67, 31.06, 29.36, 29.32, 29.22, 29.07, 29.04, 28.58, 28.09, 25.75, 23.83, 23.26, 23.14, 22.33, 31.11, 21.34, 19.67, 13.04, 12.27; HR-MS(ESI)m/z: Calcd for $C_{23}H_{47}O_2S\{[M-Cl^-]\}$, 387.3291; found, 387.3139.

*S-(4-Carboxylbutyl)-butyl-hexadecyl Sulfonium Chloride (**C4**).* Compound **B4** (0.38 g) was applied to basic hydrolysis to give **C4** (0.17 g, 0.41 mmol) as a yellowish oil, yield 48%, R_f = 0.4($MeOH-CH_2Cl_2$ = 1:9). ^1H NMR (CD_3OD, δ): 3.37−3.33 (m, 4H), 3.17 (t, J = 7.2 Hz, 2H), 2.44 (t, J = 7.1 Hz, 2H), 1.95−1.86 (m, 4H), 1.82 (dq, J = 16.3, 8.0 Hz, 4H), 1.55 (dt, J = 18.0, 8.8 Hz, 2H), 1.49 (q, J = 7.8, 7.3 Hz, 2H), 1.39 (dq, J = 31.4, 7.8 Hz, 6H), 1.38−1.26 (m, 18H), 1.04 (t, J = 7.5 Hz, 3H), 0.94 (t, J = 7.3 Hz, 3H); ^{13}C NMR (CD_3OD, δ): 171.79, 38.93, 38.82, 38.69, 38.67, 31.67, 31.07, 29.38, 29.35, 29.31, 29.21, 29.06, 29.04, 28.58, 28.10, 25.73, 23.81, 23.25, 23.15, 22.32, 21.35, 21.09, 19.67, 13.02, 12.27; HR-MS(ESI) m/z: Calcd for $C_{25}H_{51}O_2S\{[M-Cl^-]\}$, 415.3604; found, 415.3601.

SP1. PEI 1.8k (0.16 g, 3.7 mmol) was active by EDC·HCl (0.11 g, 0.55 mmol, 0.15 equiv) and NHS (0.12 g, 0.55 mmol, 0.15 equiv) and then reacted with **C1** (0.09 g, 0.37 mmol, 0.1 equiv) for 48 h. After dialysis and freeze-drying, a yellowish gel product, **SP1**, was obtained (0.23 g, yield 96%).

SP2. PEI 1.8k (0.11 g, 2.5 mmol) was active by EDC·HCl (0.07 g, 0.38 mmol, 0.38 equiv) and NHS (0.08 g, 0.38 mmol, 0.38 equiv) and then reacted with **C2** (0.08 g, 0.25 mmol, 0.1 equiv) for 48 h. After dialysis and freeze-drying, an ivory-white gel product, **SP2**, was obtained (0.14 g, yield 80%).

SP3. PEI 1.8k (0.08 g, 1.8 mmol) was active by EDC·HCl (0.05 g, 0.28 mmol, 0.15 equiv) and NHS (0.06 g, 0.28 mmol, 0.15 equiv) and then reacted with **C3** (0.0 g, 0.18 mmol, 0.1 equiv) for 48 h. After dialysis and freeze-drying, a yellowish gel product, **SP3**, was obtained (0.11 g, yield 77%).

SP4. PEI 1.8k (0.06 g, 1.3 mmol) was active by EDC·HCl (0.04 g, 0.20 mmol, 0.15 equiv) and NHS (0.04 g, 0.20 mmol, 0.15 equiv) and then reacted with **C4** (0.05 g, 0.13 mmol, 0.1 equiv) for 48 h. After dialysis and freeze-drying, a yellowish gel product, **SP4**, was obtained (0.05 g, yield 45%).

4.3. Preparation of SP/DNA Condensates and Gel Retardation. Stock SP solution was prepared at a concentration of 500 ng/μL in deionized water. The eGFP plasmid was prepared at a concentration of 100 ng/μL in water and stored at −80 °C for use. Stock SP was diluted with HEPE (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, pH = 7.5, 5 mmol/L) to form a group of SP samples at concentrations 20, 60, 120, 240, 360, and 600 ng/μL. EGFP solution (3 μL, 300 ng) was added to SP samples (5 μL). SP/DNA condensates were prepared in weight ratios of 1/3, 1/1, 2/1, 4/1, 6/1, and 10/1. The mixtures were shaken gently and cultured at 37 °C for 30 min before use.

The condensate was mixed with loading buffer containing bromophenol blue (1 μL), and then electrophoresis was carried out on the 1% (w/v) agarose gel containing ethidium bromide and Tris-acetate running buffer at 120 V for 40 min in a Sub-Cell system. DNA was visualized with a UV lamp using a Bio-Rad Universal Hood II.

The eGFP solution (3 μL, 300 ng) was added to 3 μL of SP(PEI) samples at a concentration of 200 ng/μL, forming SP/DNA condensates at weight ratios of 2:1. The mixtures were cultured at 37 °C for 30 min, DNase I (6 μL, 0.1 U/μL, a final concentration of 2 DNase I /μg DNA) was added, and the solutions were cultured for 30 min at 37 °C. The solutions were added to buffer (1 μL) containing bromophenol blue, and gel electrophoresis was performed to determine the degradation.

EDTA (3 μL, 250 mM) was added to the DNase I-treated solutions above and cultured for 30 min to stop the enzyme reaction, and then heparin sodium solution (3 μL, 6.25 U/μg DNA) was added and cultured for 30 min; the mixtures were added with buffer (1 μL) containing bromophenol blue, and gel electrophoresis was performed to determine the release of DNA.

https://doi.org/10.1021/acsomega.3c06255
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4.4. Particle Size and Zeta Potential Measurements of SP/DNA Condensates. SPs were diluted with deionized water to form samples of a concentration of 100 ng/μL. EGFP plasmid solution (8 μL, 800 ng) was added drop by drop to SP samples (16 μL) to form SP/DNA particles at a w/w ratio of 2/1. The mixtures were then shaken gently and cultured at 37 °C for 30 min. The condensates were diluted with water to 1 mL to measure particle size and zeta potential in a Nano-ZS 90 Laser Particle Size and Zeta Potential Analyzer. The measurements were carried out in the automatic mode, and the mean diameter and population distribution were computed from the diffusion coefficient using a multimodal cumulate analysis supplied by the manufacturer.

4.5. MTT Assay. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with FBS (10%, v/v) and penicillin−streptomycin (100 μg/mL) in a humidified 5% CO_2 incubator at 37 °C.

The cytotoxicity of SPs toward the HepG2 cell line was tested with MTT assays. The cells were seeded in 96-well plates at 8000 cells and 150 μL of medium per well and were cultured for 8 h to allow to attach. The medium was removed and refreshed with 150 μL of DMEM with 1% FBS. The cells were treated with 1 μL of SPs with concentrations 24, 12, 6, 3, 0.6, 0.06, and 0.006 μg/μL and were further incubated for 48 h. After incubation, the medium was removed; 90 μL of DMEM without FBS and 10 μL of MTT (5 mg/mL) were added to wells, and the cells were incubated for another 4 h. The cultured solutions were replaced with 150 μL of DMSO, and the plates were oscillated for 30 min. The absorbance of each well was measured at 490 nm using a Thermo Scientific Multiskan GO. The relative viability of the cells was calculated based on the data of five parallel tests by comparison with the controls.

4.6. Cellular Uptake Study of SP/DNA into HepG2 Cells. The eGFP plasmid was labeled with Cy5 by the Label IT Cy5 Labeling Kit according to the manufacturer's protocol. The cellular uptake of SP/Cy5-eGFP condensate was determined at a weight ratio of 2:1 on HepG2 cells and observed by a fluorescence microscope.

SP(PEI) solution (8 μL, 200 ng/μL) was diluted with DMEM to 15 μL, and Cy5-eGFP (800 ng, 15 μL) was added to the DMEM solution. The mixture was then shaken gently and cultured at 37 °C for 30 min to form the condensate of SP(PEI)/Cy5-eGFP at a ratio of 2:1.

HepG2 cells were seeded into a 24-well plate with a cell creeper, 40,000 cells/well in 500 μL DMEM with 10% FBS. The cells were incubated at 37 °C, 5% CO_2 until the cells reached 70−80% confluency. The culture media was discarded, and cells were washed with PBS. The cell medium was replaced with FBS-free culture medium (500 μL) and was added the SP(PEI)/Cy5-eGFP condensate solution (40 μL). The cells were incubated for another 4 h, and then the cell media was removed. The cells were washed with PBS (20 μL × 3) and fixed with 4% paraformaldehyde for 30 min. Afterward, paraformaldehyde solution was removed; the cells were washed three times with PBS and then 400 μL of 0.1% Triton (30 min). After removal of Triton, the cells were washed with PBS three times, and the cell creeper was sealed in a tablet with DAPI stain. The fluorescence of cells was visualized on a Leica TCS SP8 at excitation and emission wavelengths of 649/670 nm for Cy5 (red) and 364/454 nm for DAPI (blue), respectively. The cellular uptake images were analyzed for fluorescence intensity using image pro plus 6.

4.7. Gene Transfection of SP/DNA into HepG2 Cells. The SP(PEI)/eGFP condensate was prepared at a weight ratio of 2:1 by adding eGFP plasmid (250 ng) in DMEM (7.5 μL) to SP (500 ng) in DMEM (7.5 μL). The mixture was then shaken gently and cultured at 37 °C for 30 min to form the condensate. HepG2 cells were seeded into a 96-well plate, 10,000 cells/well in 150 μL, and cultured in DMEM containing 10% FBS in 37 °C 5% CO_2, until the cells reached 70−80% confluency. Before transfection, the cell culture media was dumped, and cells were washed with PBS (20 μL × 2). The cell medium was replaced with FBS in 10% culture medium (150 μL). The cell solution was added with the SP/eGFP condensate solution (20 μL) and incubated in the dark for 4 h at 37 °C. After removal of the media, the cells were washed with PBS (20 μL × 2), and fresh culture media was added with 10% FBS and cultured for another 48 h. The green fluorescence of cells was visualized on a Leica biological microscope, DMI 4000B, at excitation and emission wavelengths of 488/510 nm for green fluorescence.

4.8. DNA Release of SP/DNA Condensate by Heparin. To investigate the stability of SP/DNA, we performed a heparin release assay. PEI 25k, SP3, and SP4 were tested at a mass ratio of 2:1, and PEI 1.8k was tested at a ratio of 4:1. First, 3 μL of each solution of SP/PEI (PEI 25k, SP3, and SP4 at a concentration of 400 ng/μL and PEI 1.8k at a concentration of 800 ng/μL) was taken to EP tubes, followed by the addition of DNA solution (3 μL, 100 ng/μL). The mixtures were incubated at room temperature for 30 min. After the incubation, 3 μL of different concentrations of heparin sodium solution (0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 U/μL) were added to each EP tube to achieve final concentrations of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 U/μg DNA, respectively. The mixtures were further incubated at room temperature for 30 min. After the incubation, each sample was added 1 μL of 10X loading buffer and loaded into the gel wells to perform gel electrophoresis as described above. Naked DNA was used as a control.

4.9. Preparation and Evaluation of (SP + PEI 1.8k)/DNA Nanoparticles. SP and PEI 1.8k were diluted in HEPES buffer to a concentration of 500 ng/μL. An appropriate amount of the two solutions was taken to form a series of blended mixture, which contained PEI 1.8k at weight percentages of 50, 33, 25, and 20%. Solutions of the mixtures (6 μL, 100 ng/μL) and pDNA (3 μL, 100 ng/μL) were mixed at a weight ratio of 2:1 and incubated at room temperature for 30 min to form (SP + PEI 1.8k)/DNA condensates. These (SP + PEI 1.8k)/DNA nanoparticles were tested for heparin release and gene transfection as described in 4.7 and 4.8.

5. CONCLUSIONS

We constructed a group of hydrophobic modified low molecular weight PEI by conjugation of PEI 1.8k with trialkylated sulfonium moieties and gradually screened their abilities as a gene vector. SPs presented strong DNA compacting ability, good cell tolerance, and cell penetration. The study revealed that the strong binding effects of the sulfonium charge and the hydrophobic lipid hindered the release of DNA after cellular uptake of the nanoparticle. The balance of binding and releasing of DNA was adjusted by mixing SP with PEI 1.8k, and the mixture presented strong gene transfection. Through the experiments, the investigation of the sulfonium conjugation of PEI has presented the ability of sulfonium as a trisubstitute linkage for the discovery of the

https://doi.org/10.1021/acsomega.3c06255
ACS Omega 2024, 9, 2339−2349

new gene vectors. The convenient synthesis of sulfonium provides the possibility to build various functional groups around this cation. The substitutions of sulfonium and the conjugation ratio onto PEI need further exploration to determine a better match of structure and transfection ability.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06255.

Mass, ^{1}H NMR and ^{13}C NMR spectra of compounds **B** and **C**; IR and ^{1}H NMR spectra of SPs; and cell viability of SP3 and the mixtures of SP3 and PE I1.8k on HepG2 (PDF)

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Funding

This research was funded by the National Natural Science Foundation of China, grant number NSFC 31400660; the Heilongjiang Provincial Postdoctoral Science Foundation, grant number LBH-Q15115; and Heilongjiang "Double First Class" Construction Projects LJGXCG2022−006.

Notes

The authors declare no competing financial interest.

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https://doi.org/10.1021/acsomega.3c06255
ACS Omega 2024, 9, 2339−2349