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Construction of Peptide-Lipoic Acid Cationic Polymers with Redox Responsiveness and Low Toxicity for Gene Delivery

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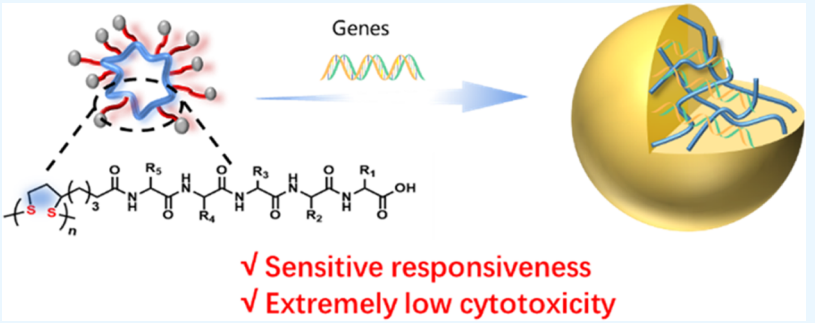

ABSTRACT: As gene therapy continues to evolve, the development of safe and effective cationic polymer carriers is critical. In this work, three polymers have been prepared by ring-opening polymerization on the basis of peptide-lipoic acid monomers. By adjusting the sequence of the peptides, redox-responsive cationic polymers with different positive charge numbers were obtained, as well as investigating their performance as gene carriers. The results showed that the polymers complexed with negatively charged genes by electrostatic interaction and successfully transported the genes into the cells, additionally degrading and releasing the genes under glutathione (GSH) conditions. Furthermore, the polymers as gene carriers in different cell lines demonstrated lower cytotoxicity, with an excellent cell survival rate of 8 times higher than the "gold standard" polyethylenimine (PEI) at the same concentration. In vitro transfection experiments showed that the polymers successfully released and transfected genes into cells, demonstrating their immense potential in gene therapy.

1. INTRODUCTION

Benefiting from modern research, gene therapy has become the most promising treatment for cancer.[1,2] Gene therapy is a method used to introduce normal functioning or therapeutic genes into targeted cells to achieve therapeutic goals.[3] However, as a negatively charged macromolecule, nucleic acid is difficult to enter cells on its own and escape degradation by nuclease, which requires the assistance of gene carriers.[4,5] Currently, the main types of vectors used in gene therapy are viral and nonviral vectors.[6] Although some viral vectors are characterized by high transduction efficiency, stable expression, and targeting to specific cells, they still face problems such as high production cost, low gene load, and high immunogenicity, which severely limit their development.[7,8] In contrast, nonviral vectors enjoy the preparation, thus possessing great potential in gene delivery.[8−10] Among the various prepared nonviral vectors,[11−16] cationic polymers have attracted tremendous attention due to their low immunoreactivity, repeatable delivery, flexible structure, and easy modification.[17] Cationic polymer vectors currently used for gene delivery include polyethylenimine (PEI), polyurethane (PAMAM), and polypropyleneimine (PPI).[18] However, most of these polymers exhibit severe cytotoxicity, extremely low transfection efficiency, and high risk of blood clotting during transfection,[19−22] which severely limits the clinical application of cationic polymer gene vectors. Therefore, it is highly desirable to develop safe and efficient strategies to deliver genes.

In recent research, it has been shown that improving the biocompatibility of gene carriers can be extremely effective in reducing their cytotoxicity, enabling them to be a safer and more effective delivery method.[23,24] Peptides are biologically active substances related to various cellular functions in living organisms and have the advantages of high purity, low toxicity, and excellent biological activity.[25−27] Currently, positively charged substances dominated by amino acids such as arginine (Arg) and lysine (Lys) show great potential for protein transport and drug delivery.[28,29] However, due to the lack of responsiveness, they consistently show a strong affinity for cargos, which greatly increases the complexity of the release

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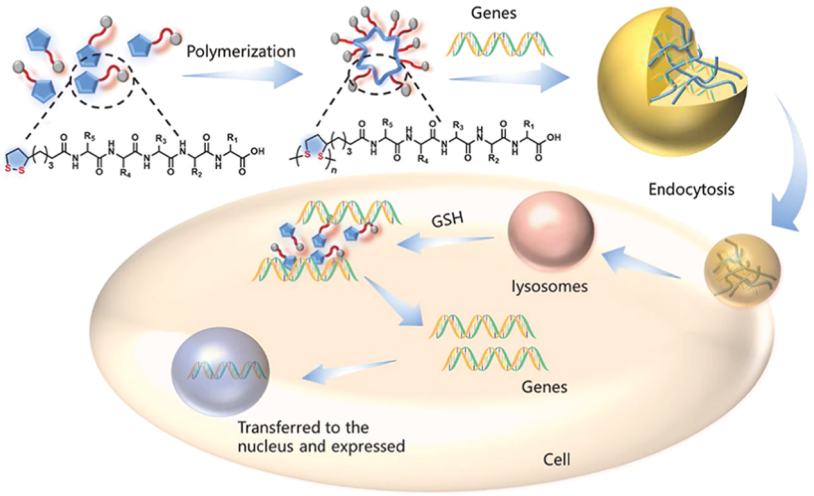
Scheme 1. Schematic Representation of the Preparation and Proposed Action Mechanism of the Nanoaggregates Through the Self-Assembly Process



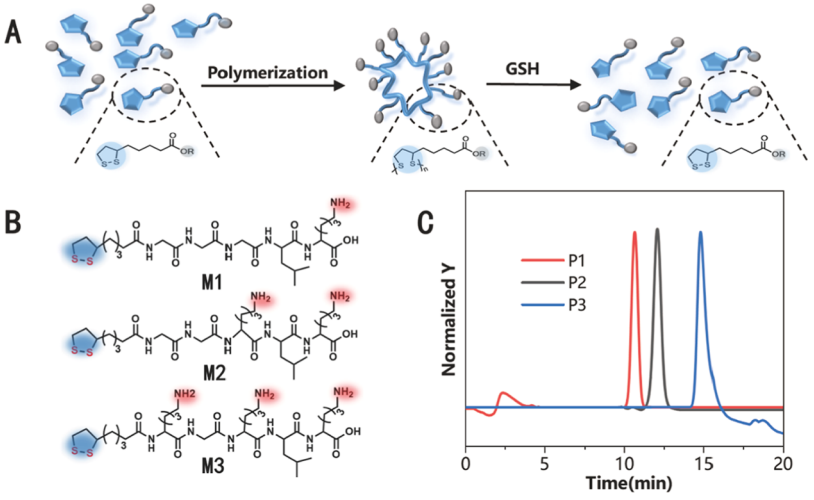


Figure 1. (A) Lipoic acid-peptide polymerization schematized. (B) Structures of three monomers of lipoic acid-peptide. (C) GPC results for polymers **P1**, **P2**, and **P3** obtained by polymerization with **M1**, **M2**, and **M3** (Mobile phase: H_2O, Flow Rate = 0.5 mL·min^{-1}).

process. Meanwhile, factors such as their nondegradability severely limit their use in biological systems. In recent years, lipoic acid and its polymers have shown good biosafety and responsiveness, demonstrating promising applications in drug and gene delivery.[29−33] The presence of disulfide bonds in poly(lipoic acid) makes it responsive to reducing environments such as glutathione, which is abundant in cells, particularly cancer cells.[34−37] Once internalized by cancer cells, the poly(lipoic acid) is rapidly degraded, releasing its components.[37] Moreover, the lipoic acid side groups have reactive sites, which makes them easy to modify.[38] Modification of lipoic acid with peptides can impart positively charged properties to the side chains of lipoic acid polymers, enabling them to bind to and deliver negatively charged biomolecules such as nucleic acids.[39] By constructing cationic polymers based on peptide lipoic acid, they have a promising application in cancer therapy and gene delivery as vectors with the advantages of sensitive response and low cytotoxicity.[40]

In this study, three peptide-lipoic acid monomers were synthesized by solid-phase synthesis.[41] A positively charged polymer, hydrophobic at one end and lipophobic at the other, was prepared by a polymerization reaction and used as a carrier for gene delivery. As shown in Scheme 1, it has a dynamic S−S bond that allows the polymer to react with glutathione (GSH) material in cells (especially tumor cells), rapidly degrading and releasing the genes it carries while maintaining a strong binding capacity. The extremely low cytotoxicity of the cationic polymers in this study compared to the "gold standard" poly(vinyl alcohol) is due to the fact that the polymers prepared in this study were synthesized from highly biocompatible lipoic acid and peptides. This study demonstrates the feasibility of these polymers for gene delivery and their great potential as multifunctional biomolecular delivery vehicles. In addition, depending on the reactive sites of the lipoic acid side chains, the polymers can be endowed with different functions by adjusting the side chain sequences, resulting in the preparation of various polymeric functional materials. By construction of cationic polymers based on peptide lipoic acid, many candidate gene delivery vectors with sensitive responses and low cytotoxicity can be prepared, which have broad application prospects in cancer therapy and gene delivery.

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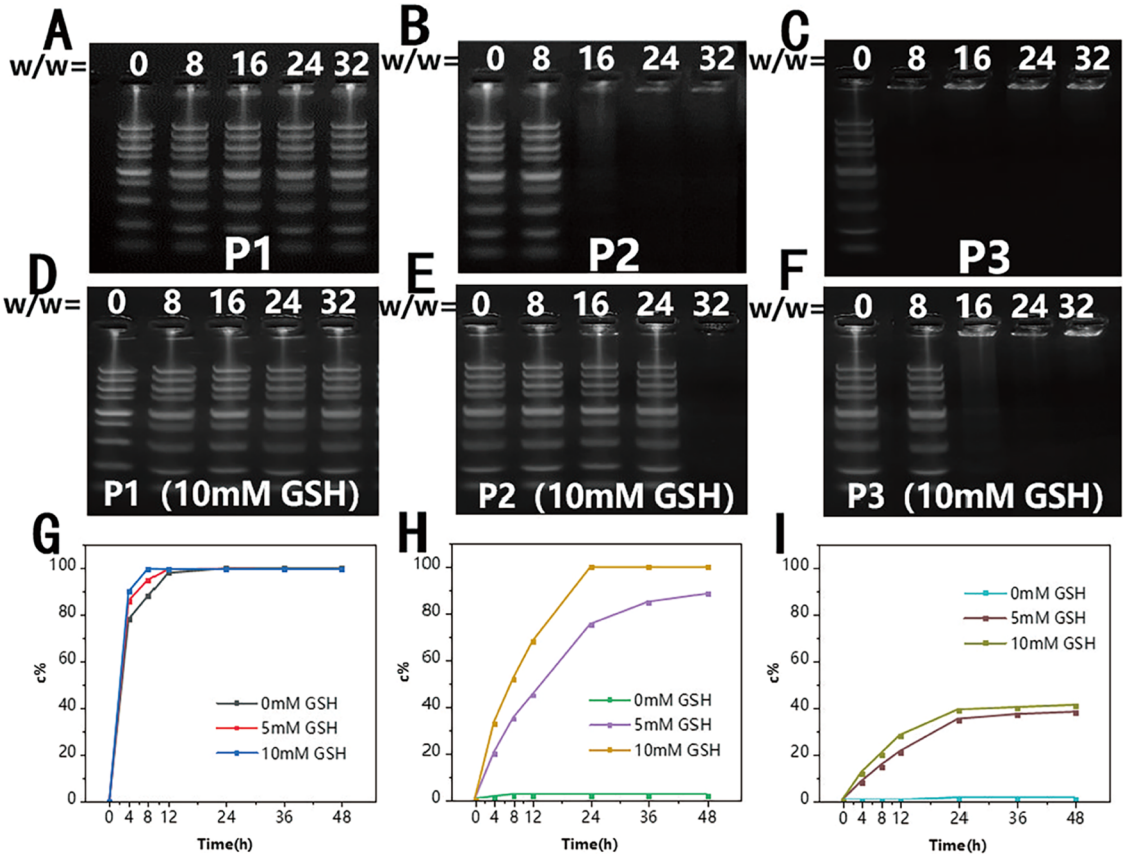

Figure 2. (A−C) Agarose gel electrophoresis (AGE) test, Polymer to DNA ratio from w/w = 8/1 to 32/1. (D−F) Agarose gel electrophoresis (AGE) test, Polymer to DNA ratio from w/w = 8/1 to 32/1 with 10 mM GSH. (G−I) DNA release from complex formed by polymer **P1, P2, and P3** with DNA at w/w = 24/1 at different GSH concentrations.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization of Target Polymers. To obtain a low-toxicity lipoic acid polymer while ensuring that the polymer could be degraded in a reducing environment and meet the requirements of the release genes (Figure 1A), we used peptides to modify the lipoic acid monomer. In order to provide the prepared polymers with a positive charge, we incorporated different amounts of Lys into the design of the peptide structure. In addition, we chose glycine (Gly) to take advantage of its small spatial volume and flexibility to reduce the spatial resistance of the polymers and improve the adaptability of the polymers to different spatial conformations. Meanwhile, to prevent the polymer from self-assembling directly in aqueous solution, we inserted leucine (Leu) to block the structural regularity of its side groups. From this, we designed three pentapeptides with sequences of (i) Lys-Leu-Gly-Gly-Gly, (ii) Lys-Leu-Lys-Gly-Gly, and (iii) Lys-Leu-Lys-Gly-Lys.

By grafting to the lipoic acid side group, we finally obtained three monomers, as shown in Figure 1B. The resulting monomers were analyzed by mass spectrometry, and the detected molecular weights were consistent with those of our target monomers as shown in Figures S1−S3. These results indicate that the peptide-lipoic acid monomers with different charges were successfully synthesized. By anionic ring-opening polymerization, we obtained three different polymers **P1−3**. We performed GPC analysis as well as ^{1}H NMR analysis of the polymers and the results are shown in Figure 1C, Table S1, and Figures S4−S6. These results confirmed the successful preparation of the polymers. In conclusion, we obtained three sequence-tunable poly(lipoic acid-peptide) monomers by solid-phase synthesis and successfully prepared sequence-tunable cationic polymers by anionic polymerization. In addition, cationic polymers with good protonation capabilities not only increase surface charge specifically in pathological environments but also enable effective in vivo escape via proton sponging. In order to study the protonation ability of different polymers, pH titration experiments were carried out. As can be seen in Figure S7, the protonation capacities of the polypeptide sequences are different depending on their sequences. This experimental result proved that by modulating the peptide sequences, it is indeed possible to change its proton buffering capacity and buffer interval and to realize effective and controllable protonation. And among our polymers, **P2** and **P3** have the best protonation ability, which is favorable for plasmid transfection and expression.

2.2. Interaction of Polymers with DNA. The ability of cationic polymers to efficiently bind and release nucleic acids is important for gene delivery. Since DNA bound to cationic polymers is not easily released by electrophoresis, the polymer-gene binding ability can be evaluated by AGE of complexes with different w/w (polymer/DNA mass ratio). DNA marker was used to verify the loading capability of cationic polymer supports for genes containing different numbers of base pairs. As shown in Figure 2A, the DNA was released by electrophoresis regardless of the weight ratio between the polymer **P1** and the DNA, indicating the limited ability of **P1** to capture DNA. In contrast, when the mass ratio reached 16/

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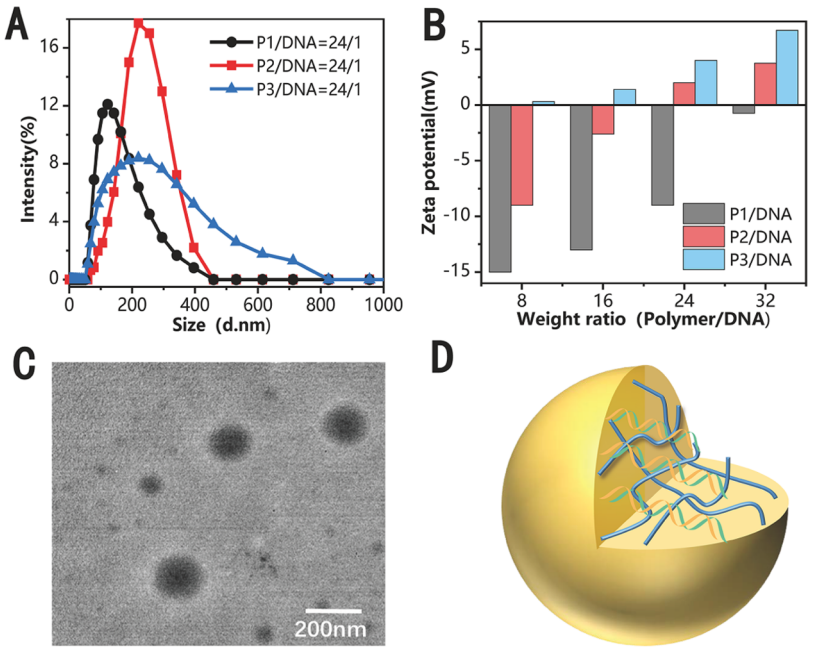

Figure 3. (A) Size distribution of the complex formed by polymer with DNA is at w/w = 24/1. (B) The ζ potentials of the complex (from w/w = 8/1 to w/w = 32/1). (C) TEM images of the complex formed by polymer P2 with DNA at a w/w = 24/1. (D) Schematic diagram of mixture.

1, both polymers **P2** and **P3** successfully entrapped genes with different base pair numbers (Figure 2B,2C), with **P3** entrapping better. These results indicate that polymers **P2** and **P3** can effectively compress and load DNA, and their ability increases with increasing charge number.

In order to test the ability of the cationic polymers to release the cargo again, the complexes formed by the polymer with the DNA were exposed to a 10 mM GSH solution and then analyzed with AGE. As shown in Figure 2D−F, the electrophoretic results of the complex formed by polymer **P1** with DNA did not change significantly after the addition of the GSH reaction. Meanwhile, the complex formed by polymer **P2** with DNA has a tendency to release DNA, but the release is not obvious at the highest mass ratio. The results also showed that the complexes formed by polymer **P3** with DNA significantly released DNA at w/w = 8, but the ability to release was not significant as the mass ratio increased. This suggests that the presence of GSH enhances the ability of **P2** and **P3** to release DNA to some extent.

We used a microspectrophotometer to measure the concentration of DNA to characterize its release and verify the release of DNA from the complex under GSH conditions. We selected the complexes with a mass ratio of 24/1 as representative for testing. As shown in Figure 2G−I, in the absence of GSH, the complexes formed by polymers **P2**, **P3** with DNA can hardly release DNA and are stable for more than 48 h. However, when 5 mM GSH solution is added for 24 h, the complex formed by polymer **P2** with NDA released more than 60% of DNA. Furthermore, when the GSH concentration was increased to 10 mM, the release of DNA reached 100%. In contrast, less than 40% of the DNA was released from the complex formed by polymer **P3** with DNA even after 24 h of addition of a 10 mM GSH solution. The differences in release effects prompted our thinking. To investigate the effect of different complexes on release, we performed AGE analyses on monomer/DNA complexes with different mass ratios (Figure S8). As expected, **M1** was not significantly bound to DNA. It is noteworthy that at w/w (monomer/DNA mass ratio) = 24/1, **M2** showed weaker interception of DNA, whereas **M3** exhibited an excellent interception effect on DNA. These explain to some extent the better effect of the complex formed by polymer **P2** with DNA on gene release after depolymerization, whereas the complex formed by polymer **P3** with DNA has an intercepting effect even after depolymerization and a poorer effect on gene release.

These results indicated that **P1** exhibited the poorest loading of DNA, and **P3** possessed the best loading of DNA, but it had an average release effect. In contrast, **P2** demonstrated excellent results in both the loading and release of DNA. In addition, the complexes formed by polymers **P2** and **P3** with DNA are barely released within 48 h, ensuring that the complexes are stable during transfection. At the same time, the disulfide bonds in the complex are highly sensitive to GSH, which can depolymerize the complex and release the DNA. As the expression of GSH is high in most cells, especially tumor and cancer cells, this would be very favorable for the release of DNA. Therefore, polymer **P2** is a promising gene delivery vector with potential applications in antitumor gene therapy.

2.3. Characterization of Polymer with Plasmid Complexes. The particle size distribution and ζ potential of the complexes formed by the polymer with the **pAAV-CAG-GFP** plasmid (pDNA) were determined by DLS. As shown in Figures 3A and S9, we found that all the complexes were effective in concentrating the plasmid to form nanoparticles in the range of 100−400 nm. Among them, the complexes formed by polymer **P2** with plasmid have the most uniform size distribution. On the other hand, ζ potential tests showed that the potential at the surface of the complex increased with increasing mass ratio. At w/w = 24/1, the complex formed by polymer **P2** with plasmid becomes positively charged (Figure 3B), in agreement with the AGE results. The results also showed that polymer **P1** formed the lowest potential complex with the plasmid, resulting in the poorest binding to the

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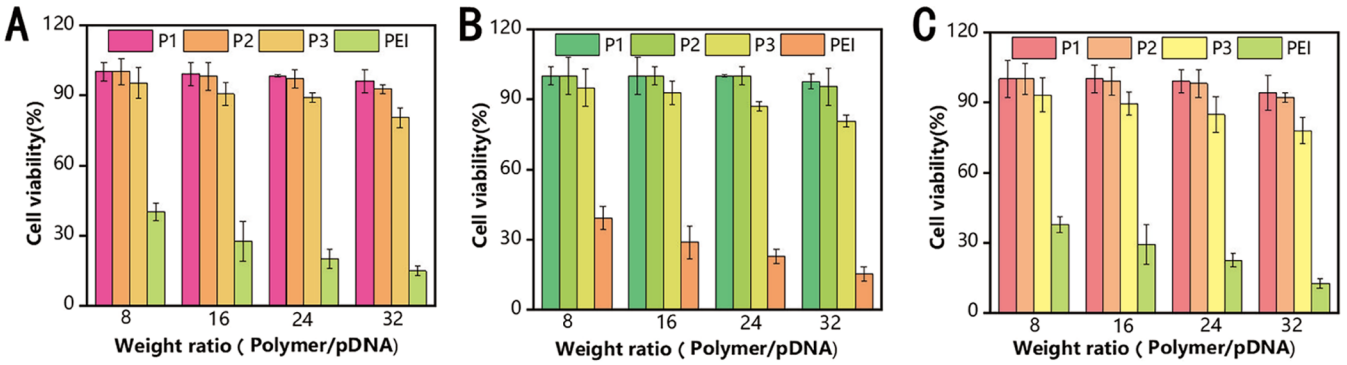

Figure 4. Viability data of different cell lines exposed to different polymer/pDNA weight ratios: (A) 293T cells. (B) CHO-K1 cells. (C) Hela cells.

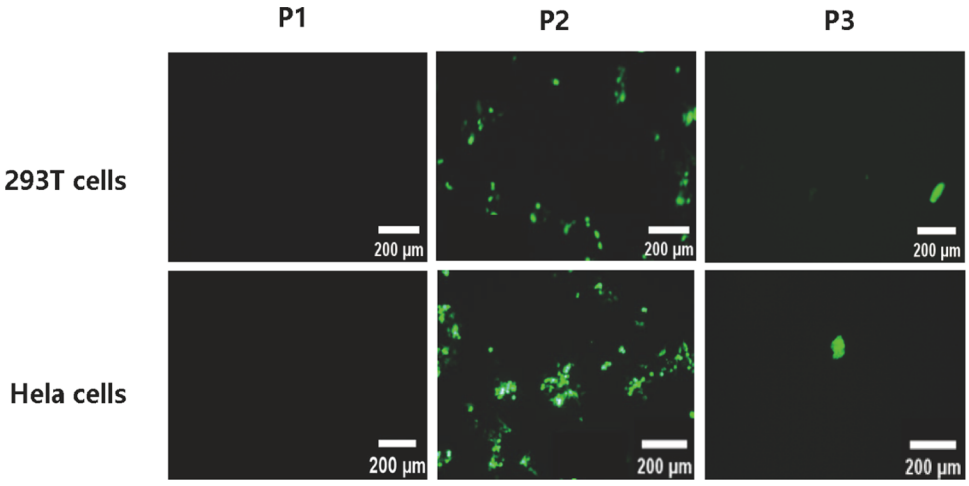

Figure 5. Transfection ability of the three mixtures in cells was observed by fluorescence microscopy at Polymer/pDNA = 24.

plasmid. In contrast, the complex formed by polymer **P3** with the plasmid has the highest potential, consistent with its strong interception effect. The positive potential on the surface of the complexes would favor their entry into the cell by electrostatic interactions with the negatively charged phospholipid bilayer.

In addition, the morphology of the polymers and complexes in this study was observed by using transmission electron microscopy (TEM). The results of the images in Figure S10 show that the polymers did not form regular shapes when left alone, which is consistent with our expected nonself-assembly behavior of the polymers. However, after binding to the plasmid, the complexes can be seen as clearly regular spherical complexes. The complex formed by polymer **P2** with a plasmid at w/w = 24/1, observed by TEM for the morphology, is used as an example. The results of the images in Figure 3C show that polymer **P2** is able to concentrate with the plasmid in deionized water to form regular spherical nanoparticles without aggregation, suggesting that they are well stabilized and will be very beneficial in the delivery process. According to these tests, our polymers and genes were complexed together by electrostatic interaction to form spherical nanoparticles, from which we hypothesized that the complex morphology could be as shown in Figure 3D.

2.4. Cytotoxicity. We analyzed the cytotoxicity of different polymer/pDNA complexes using the CCK-8 assay in different cell lines such as 293T cells, CHO-K1 cells, and Hela cells with PEI (25 kDa) as a control, and the results are shown in Figure 4. In the complexes formed by PEI and the plasmid, the survival rate of surviving cells in the cell lines was very low. Fortunately, however, all three vectors we synthesized showed low cytotoxicity with at least 50% of the cells surviving even at high mass ratios. Polymers **P1** and **P2** showed the lowest cytotoxicity with more than 80% of the cells surviving. The results indicate that our cationic polymer carriers have higher cell viability, good biocompatibility, and very low cytotoxicity compared to the same concentration of polyethyleneimine. These properties reduced the cellular immunogenicity of the cationic polymer, made it easily free from lysosomal degradation, and reduced the risk of coagulation during gene delivery. Therefore, the cationic polymers prepared in this study can be used more safely and efficiently as gene delivery vectors, which is very important for gene therapy and has a wide range of applications.

2.5. In Vitro Gene Transfection. To verify whether the plasmid could be successfully released and efficiently expressed in cells, we performed transfection experiments to detect the expression of the green fluorescent protein (GFP). In this study, we chose pAAV-CAG-GFP, which is a plasmid that can stably express green fluorescent protein in conventional mammalian cell lines, and it is suitable for the 293T cells and Hela cells we used; it is also beneficial for observation and analysis. We observed the transfection effect of different ratios of the three cationic polymers, as shown in Figures 5 and S11. The results showed that the transfection effect of the complex formed by polymer **P1** and plasmid was not satisfactory, and the presence of green fluorescent protein was almost not observed in the field of view of all mass ratios, which indicated that the plasmid was not successfully expressed. This may be

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due to the limited ability of **P1** to capture the plasmid and the negative potential on the surface of the complex. Similarly, for the transfection effect of the complex formed by polymer **P3** with the plasmid, we observed very dispersed and few green fluorescent proteins under the microscope, suggesting that **P3**, although it has a certain transfection ability, was not expressed satisfactorily due to its limited gene release ability.

In contrast, the complex formed by polymer **P2** with the plasmid (w/w = 24/1) was transfected well, and green fluorescent protein was observed throughout the transfection process. This suggests that the expression of the plasmid was successful and that **P2** has a good ability to carry and release genes at this ratio. Meanwhile, we found that the complex was more satisfactorily expressed in Hela cells and flow cytometry analysis confirmed this conjecture. In conclusion, the ability of peptide-lipoic acid polymers to carry and release DNA is affected by the number of side group charges, and they can carry DNA during endocytosis, successfully transfect plasmids into cells, and release plasmids for expression in cells. However, the transfection efficiency of these polymers must be further improved. In the future, how to utilize peptide-lipoic acid polymers for safe and efficient gene delivery will be our focus.

3. CONCLUSIONS

In order to find safe and effective cationic polymer gene delivery carriers, in this study, three peptides with different charge numbers were synthesized by solid phase synthesis and three peptide-lipoic acid monomers were obtained by peptide modification of lipoic acid side groups. Then, three cationic polymers with different positive charge numbers were obtained by anionic ring-opening polymerization. These polymers can be assembled with negatively charged genes via the positively charged groups of the peptide side chain to form regular spherical nanoparticles that deliver genes into cells. In particular, the polymer, which is composed of lipoic acid and peptides, is highly biocompatible and has a much better cytotoxicity than existing vectors. Experimental results show that the cell survival rate of the newly developed cationic polymer is 8 times higher than that of PEI at the same concentration, which is important for the development of safe and effective gene vectors. The disulfide bonds in the complexes formed by the polymer complexed with the genes are stable under normal physiological conditions but degrade and release the genes in reducing environments, offering great potential for gene delivery. We found that differences in the amino acid sequences of short peptides modulate the number of positive charges carried by cationic polymers and that such differences can affect the capture and release capabilities of gene vectors, thereby greatly influencing transfection efficiency. These properties give the short peptide-lipoic acid-based cationic polymers ideal gene delivery properties with the advantages of low cytotoxicity and environmental sensitivity, and they are expected to be widely used as gene delivery platforms for gene therapy.

4. EXPERIMENTAL SECTION

4.1. Materials and Characterization. The materials used in this study and their characterization are described in the Supporting Information.

4.2. Synthesis of Monomers and Polymerization of Peptide-Lipoic Acid. In this study, the monomers were synthesized by using the solid phase synthesis method. The peptides were synthesized on Wang resin using solid-phase synthesis.[41] The sequences were: (i) Lysine (Lys)- Leucine (Leu)- Glycine (Gly), (ii) Lys-Leu-Lys-Gly-Gly, and (iii) Lys-Leu-Lys-Gly-Lys. The reaction was performed using benzotriazole- *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT), and *N*,*N*-diisopropylethylamine (DIEA). The reaction was carried out according to amino acid (AA)/HBTU/HOBT/DIEA = 3:3:3:4. The solvent in the reaction was DMF or dichloromethane (DCM) for more than 40 min. After completion of the coupling, the excess amino group was closed with acetic anhydride/pyridine = 1:1 for 15 min. The amino protecting groups of the terminal amino acids were then removed with 20% piperidine for 30 min, and the completion of deprotection was tested with ninhydrin reagent. Lipoic acid was grafted to the peptide using 3 equiv of TA, 3 equiv of HBTU, and 4 equiv of DIEA coupled to the peptide termini. Each step of the reaction was washed with DCM and DMF upon completion. The resin was treated with the lysis solution trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) = 99:0.5:0.5 for 3 h. The mixture solution was then precipitated with ether. The resulting white solid was dissolved in an aqueous solution and purified by reversed-phase HPLC (water/acetonitrile plus 0.1% TFA) using a C18 preparative scale column to obtain the monomer of the Lipoic acid-peptide. The resulting monomers were analyzed by mass spectrometry.

The monomer was polymerized by anionic ring-opening polymerization.[42] 100 mg of monomer was configured with H_2O at a concentration of 1 M. The polymerization reaction was carried out at room temperature with stirring while the reaction system consisted of monomer/1,8-diazabicyclo [5.4.0] undecane-7 (DBU)/benzyl mercaptan (PhSH) = 20:1:1. After 1.5 h, the reaction is quenched by adding excess phenyl isocyanate (PhNCO) for 30 min. The reaction is then purified with anhydrous ether and dialyzed in ultrapure water for 5 days using a dialysis bag with a molecular retention capacity of 1000 and changing the ultrapure water twice a day. The cationic polymer is lyophilized.

4.3. Polymer pH Titration Analysis. In order to study the protonation ability of different polymers, a PH titration experiment was performed. Twenty mg of each of the three polymers were dissolved in 3 mL of 0.1 mol/L H_2O acid and fixed with water to 20 mL, then titrated with 0.01 mol/L NaOH. The change in pH of the solutions was detected using a PH meter and then plotted on a graph and pK_a was calculated.

4.4. Agarose Gel Electrophoresis (AGE) Assay. Monomers **M1**, **M2**, and **M3** and polymers **P1**, **P2**, and **P3** were prepared as polymer solutions by dissolving 5 mg each in 1 mL of water and complexing with DNA marker according to w/w (polymer or monomer mass/gene mass) = 8/1 to w/w = 32/1 and shaking for 30 min. The complexes were then loaded onto agarose gels containing GEL GREEN nucleic acid dye. The gels were separated by electrophoresis at 120 V in a TAE buffer for 20 min, then visualized and recorded. For reducing environment response experiments, the complexed samples were dispersed in a 10 mM GSH solution, shaken at 37 °C for 24 h under nitrogen protection and then subjected to agarose gel electrophoresis as described above.

4.5. DNA Release Experiments. The amount of DNA released from the polymer/DNA complexes at different GSH concentrations was determined by using a DNA concentration

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detector. The complexes were shaken at 37 °C for different periods of time under nitrogen protection at 0 mM GSH, 5 mM GSH, and 10 mM GSH, and a control group containing only DNA was also set up. The complexes were then centrifuged in a high-speed centrifuge for 10 min (rpm = 12,000 rpm), and the supernatant was collected to determine the concentration of DNA using a microspectrophotometer.

4.6. Dynamic Light Scattering (DLS) Assay. Polymer solutions were prepared by combining the three polymers in ultrapure water, complexed with the pAAV-CAG-GFP plasmid according to w/w = 24/1, and shaken for 30 min. The hydrodynamic diameters and ζ potentials of their dispersions in ultrapure water at 25 °C were then measured using a DLS instrument.

4.7. Cytotoxicity. Cells were homogeneously inoculated into a 96-well plate containing 200 μL of complete medium per well at an inoculation volume of 1×10^4 cells/well and cultured in a cell culture incubator until 80% of the cells had grown and fused. Using 500 ng of plasmid as a standard, the original medium was replaced with medium containing different mass ratios of polymer/pDNA complex, and a control group was established, one group did no preparation and only replaced the medium, and the other group replaced the polymer with PEI (25 kDa). Four replicate wells were used per group. After 24 h of incubation, 10 μL CCK-8 working solution was added to each well and the cells were incubated in a cell culture incubator for 2.5 h. After sufficient color development, the absorbance at 450 nm was measured with an enzyme marker to calculate cell viability.

4.8. Gene Transfection Experiments. The polymer solution was removed and complexed with the pAAV-CAG-GFP plasmid at ratios of w/w = 8/1 to 32/1 under sonication conditions. Cells are inoculated into culture dishes and cultured in a complete medium. After the cells reached 60−80% fusion, complete medium was replaced with 500 μL of Opti-MEM medium containing pAAV-CAG-GFP plasmid in poly(lipoic acid peptide) mix and incubated at 37 °C for 4 h with PEI (25 kDa) at w/w = 8 as a positive control. The cells were incubated with the complete medium for another 24 h. The cells were observed using a fluorescence inverted microscope and subsequently treated with trypsin, and the positivity of GFP expression in each group was detected by flow cytometry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c07194.

Details of mass spectrometry results, NMR analyses, PH titration curves, agarose gel experiments, particle size distributions, scanning electron microscope images, transfection results, and experimental materials and apparatus (PDF)

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Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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