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A Universal GSH-Responsive Nanoplatfor for the Delivery of DNA, mRNA, and Cas9/sgRNA Ribonucleoprotein

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Abstract

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ASSOCIATED CONTENT

Supporting Information

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GPC analyses and ¹H NMR spectra of the polymers; DLS analyses and TEM images of the polyplexes; stability of the DNA polyplexes; histograms of the mean GFP fluorescence intensity in the DNA transfection assay; and transfection of DNA polyplexes in HEK 293 cell spheroids (PDF)

The long-sought promise of gene therapy for the treatment of human diseases remains unfulfilled, largely hindered by the lack of an efficient and safe delivery vehicle. In this study, we have developed a universal glutathione-responsive nanoplatform for the efficient delivery of negatively charged genetic biomacromolecules. The cationic block copolymer, poly(aspartic acid-(2-aminoethyl disulfide)-(4-imidazolecarboxylic acid))–poly(ethylene glycol), bearing imidazole residues and disulfide bonds, can form polyplexes with negatively charged DNA, mRNA, and Cas9/sgRNA ribonucleoprotein (RNP) through electrostatic interactions, which enable efficient cellular uptake, endosomal escape, and cytosol unpacking of the payloads. To facilitate the nuclear transport of DNA and RNP, the nuclear localization signal peptide was integrated into the DNA or RNP polyplexes. All three polyplex systems were fully characterized and optimized in vitro. Their relatively high transfection efficiency and low cytotoxicity, as well as convenient surface functionalization merit further investigation.

Keywords

redox-responsive polymer; polyplex; gene editing; gene therapy; nonviral vehicle

1. INTRODUCTION

Gene therapy has attracted increasing attention due to its great potential to treat or prevent human diseases, such as cancers and genetic disorders.^{1–3} DNA and mRNA are among the most commonly used therapeutic nucleic acids. DNA is used to introduce new copies of genes into target cells and require transfer into the cell nucleus.⁴ On the other hand, mRNA provides a full template for the expression of the protein of interest in the cytoplasm without the need to enter the nucleus to function.⁵ More recently, CRISPR-Cas9/sgRNA ribonucleoprotein (RNP) has been developed and is now considered an easy-to-use, efficient, and precise genome editing tool for gene therapy.^{6,7}

The success of gene therapy is critically dependent on the strategies to protect these negatively charged therapeutic genetic biomacromolecules and deliver them to the desired subcellular space (e.g., the nucleus for DNA and Cas9 RNP and the cytosol for mRNA).⁸ However, the lack of efficient and safe gene delivery methods still presents a critical obstacle to the routine clinical implementation of human gene therapy.^{9,10} Although viral vectors offer high delivery efficiency, their severe drawbacks, including a high risk of immunogenicity, safety concerns, and complicated production processes, largely limit their clinical usage.^{11,12} Nonviral vectors, including cationic lipids, cationic polymers, and functionalized inorganic nanoparticles, have been widely studied and have emerged as a safer, less expensive, and easier-to-produce alternative to viral vectors.^{12,13} Nevertheless, the use of nonviral vectors is still largely limited by their relatively lower transfection efficiency in comparison with viral vectors.^{14,15}

Hence, there is still a great need to engineer efficient and safe nonviral platforms for genetic biomacromolecules. Cationic polymer-based systems have been actively investigated for gene therapy owing to their diversity in terms of polymer structures, compositions, and functional groups.^{16–18} Cationic polymers can interact with negatively charged genetic

materials via electrostatic interactions to form polyplexes.¹⁹ To improve their transfection efficiency, several factors need to be taken into consideration: (1) efficient cellular uptake; (2) rapid endosomal escape to prevent degradation; (3) efficient payload decomplexation from polyplexes in the cytosol; and (4) nucleus transport for certain payloads, such as DNA and RNP. To fulfill these strict criteria, one of the most promising and possibly indispensable strategies is to use certain biological triggers, including pH changes and the presence of certain molecules or enzymes inside cells.^{20–22} The most widespread theory used to achieve sufficient endosomal escape is the proton sponge effect.^{23,24} Imidazole groups, ($pK_a \sim 6.0$) possessing a large proton buffering capacity in the acidic endocytic compartments, have been frequently incorporated into polymeric vectors to enable endosomal escape, thus enhancing delivery efficiency.^{16,25} Another commonly used biological trigger is the presence of highly concentrated glutathione (GSH) (2–10 mM) in the cytosol, whereas the concentration of GSH extracellularly is approximately 1000-fold lower (2–20 μM).²⁶ Disulfide bonds can be selectively cleaved in the cytosol due to the high concentration of GSH, which has been widely utilized to engineer a rapid cytosol release of the payloads from the nanocarriers.^{26–28}

In this study, we have developed a universal cationic block copolymer capable of delivering negatively charged genetic biomacromolecules, such as DNA, mRNA, and Cas9 RNP. The cationic block copolymer, poly(aspartic acid-(2-aminoethyl disulfide)-(4-imidazolecarboxylic acid))–poly(ethylene glycol) (i.e., P(Asp-AED-ICA)–PEG), bearing imidazole residues and disulfide bonds, can form polyplexes with negatively charged DNA, mRNA, and Cas9 RNP through electrostatic interactions (Figure 1). PEGylation can offer a neutral surface charge, potentially enhance the stability of the polyplexes, and allow for versatile ligand conjugations of the polyplexes. The imidazole residues provide the polyplexes with a rapid endosomal escape capability. Once in the cytosol, the pendent –S–S– bond will be cleaved, which converts the cationic polymers to neutral polymers, thus releasing its payload (i.e., DNA, mRNA, or Cas9 RNP) from the polyplexes. Moreover, to facilitate nuclear transport of DNA, DNA was complexed to the positively charged nuclear localization signal (NLS) peptides before forming DNA polyplexes. For RNP, Cas9 proteins originally tagged with two NLS peptides were used for RNP polyplexes.^{29,30} All three polyplex systems were successfully prepared and optimized in vitro in terms of transfection efficiency and cytotoxicity. Their relatively high efficiency and low cytotoxicity warrant further studies for both in vitro and in vivo applications.

2 MATERIALS AND METHODS

2.1 Materials.

β -Benzyl L-aspartate *N*-carboxyanhydride (i.e., BLA-NCA) was prepared as previously reported.³¹ Methoxy–PEG–NH₂ (PEG–NH₂, $M_n = 5$ kDa) was obtained from JenKem Technology (Allen, TX). 4-Imidazolecarboxylic acid was obtained from Sigma–Aldrich (St. Louis, MO). The sNLS-Cas9-sNLS was obtained from Aldevron (Madison, WI). The NLS peptide (amino acid sequence: PKKKKRKV) was synthesized by Abi Scientific Inc. (Sterling, VA). Other reagents were obtained from Thermo Fisher Scientific (Fitchburg, WI).

2.2 Preparation of Poly(β -benzyl L-aspartate)–Poly(ethylene glycol) (i.e., PBLA–PEG).

BLA-NCA (106 mg) and PEG–NH₂ (50 mg) were dissolved in dimethylformamide (5 mL). The reaction was performed at 55 °C for 48 h. The resulting mixture was added dropwise into a 10-fold volume of cold diethyl ether. The precipitate was then collected, washed with diethyl ether once, and dried under vacuum.

2.3 Synthesis of Poly(aspartic acid-(2-aminoethyl disulfide))–Poly(ethylene glycol) (i.e., P(Asp-AED)–PEG).

2-Aminoethyl disulfide (13.1 mg) and PBLA–PEG (20 mg) were dissolved in dimethyl sulfoxide (DMSO) (10 mL). The reaction was performed at room temperature (i.e., RT) for 24 h. Thereafter, the resulting solution was dialyzed (molecular weight cutoff: 15 kDa) against deionized (DI) water for 48 h. The product was obtained after freeze-drying.

2.4 Synthesis of Poly(aspartic acid-(2-aminoethyl disulfide)-(4-imidazolecarboxylic acid))–Poly(ethylene glycol) (i.e., P(Asp-AED-ICA)–PEG).

4-Imidazolecarboxylic acid (0.57 mg), P(Asp-AED)–PEG (20 mg), 1,3-dicyclohexylcarbodiimide (4.4 mg), and *N*-hydroxysuccinimide (2.9 mg) were dissolved in DMSO (5 mL). The reaction was performed at RT for 24 h. The resulting solution was then dialyzed (molecular weight cutoff: 15 kDa) against DI water for 48 h. The product was obtained after freeze-drying.

2.5 Preparation of DNA Polyplexes, mRNA Polyplexes, and RNP Polyplexes.

To prepare DNA polyplexes, an appropriate amount of the P(Asp-AED-ICA)–PEG polymer was added to pDNA (250 ng) phosphate-buffered saline (PBS) solutions to obtain a specified N/P ratio (the molar ratio of the amine groups of cationic polymers to the phosphate groups of DNA) that were then incubated at RT for 25 min. An agarose gel retardation electrophoresis assay was carried out to determine the minimum N/P ratio between polymers and pDNA for a complete complexation. Electrophoresis was performed on 1% agarose gel in a Tris–acetate–ethylenediaminetetra-acetic acid (EDTA) buffer solution (100 mL) containing SYBR Safe DNA Gel Stain (10 μ L; Thermo Fisher, Fitchburg, WI) with a current of 100 V for 40 min on a Mini-Sub Cell GT horizontal electrophoresis system (Hercules, CA). The final DNA concentration was 200 ng per well. The retardation of the DNA polyplexes was visualized on a UV illuminator (Bio-Rad, Inc., Hercules, CA). mRNA polyplexes and RNP polyplexes were prepared following a similar protocol.

2.6 Characterization.

¹H NMR spectra of polymers were recorded on an NMR spectrometer (Varian Mercury Plus 300 MHz), using DMSO-*d*₆ or CDCl₃ as a solvent. Molecular weights (both M_n and M_w) and polydispersity indices (PDI) of all of the polymer products were measured by a gel permeation chromatography (GPC; Viscotek) system equipped with triple detectors (a refractive index detector, a viscometer detector, and a light scattering detector). The morphologies of the polyplexes were studied by a ZetaSizer Nano ZS90 dynamic light scattering (DLS; Malvern Instruments; 0.5 mg/mL) and transmission electron microscopy (TEM; FEI Tecnai G² F30 TWIN 300 KV, E.A. Fischione Instruments, Inc.). To prepare

TEM samples, a drop of the polyplex solution (0.05 mg/mL) was deposited onto a 200 mesh carbon-coated copper grid and dried at RT. The samples were further stained by 1 wt % of phosphotungstic acid.

2.7 Cell Culture.

HEK 293 cells (a human kidney embryo cell line), MZ-CRC-1 cells (a human neuroendocrine cancer cell line), RAW 264.7 cells (a mouse monocyte cell line), and U87-MG cells (a human brain cancer cell line) were cultured with a complete medium containing 89% Dulbecco's Modified Eagle Medium, 10% fetal bovine serum (FBS), and 1% penicillin–streptomycin under standard conditions (37 °C/5% CO₂/95% humidity). HCT 116 cells (a human colon cancer cell line) were cultured with 89% McCoy's 5A medium, 10% FBS, and 1% penicillin–streptomycin. All reagents were obtained from Gibco (Gaithersburg, MD).

2.8 DNA Polyplex Transfection.

DNA transfection efficiency was tested in HEK 293, MZ-CRC-1, U87-MG, and HCT 116 cells. Enhanced green fluorescent protein (EGFP) plasmid DNA (Addgene, Cambridge, MA; 250 ng per well) was used for this study. Cells were seeded in a 96-well plate (20 000/well) and were cultured overnight. Cells were then treated with one of the following: (1) pure media as a negative control; (2) Lipofectamine 2000/DNA (i.e., Lipo2000; Thermo Fisher, Fitchburg, WI) as a positive control following the manufacturer's protocol (0.4 μ L of Lipo2000 per well); or (3) DNA polyplex with different N/P ratios (i.e., 10, 20, and 30) and different amounts of NLS peptides (N/P = 0, 0.25, and 0.5). After incubation for 48 h, cells were harvested with 0.25% EDTA–trypsin (Gibco, Gaithersburg, MD), spun down (5 min, 130 $\times$ g), and suspended in PBS (500 μ L) for flow cytometric analyses that were carried out on a flow cytometer (Attune NxT; Thermo Fisher, Fitchburg, WI). Data were analyzed using FlowJo 7.6 (Ashland, OR). All experiments were performed in triplicate. The transfection efficiency of the DNA polyplexes was determined by the percentage of GFP positive cells in the cells treated with DNA polyplexes or Lipo2000/DNA. Cells treated with pure media were used to define the gate for GFP negative cells.

To study the stability of the DNA polyplexes in the presence of GSH, the transfection efficiency of the DNA polyplexes under different GSH concentrations was tested. Transfection experiments were performed under similar conditions using GSH-containing media instead. The GSH concentration investigated ranged from 0.001 to 20 mM.

2.9 Intracellular Trafficking of DNA Polyplexes.

HEK 293 cells were seeded in an 8-well chamber slide (20 000/well) and were cultured overnight. Cells were treated with DNA polyplexes (N/P ratio of 20) with or without NLS. Cy3.5 DNA was used for intracellular tracking. At certain time points (i.e., 0.5, 2, and 6 h), the cells were washed three times with PBS and were then stained with LysoTracker Green DND-26 (100 nM) for endosomes/lysosomes and Hoechst (5 ng/mL) for the nuclei, at 37 °C for 20 min. Cells were then washed three times with PBS. The cellular localization of DNA was visualized with a confocal laser scanning microscope (i.e., CLSM, Nikon Eclipse Ti inverted microscope with Nikon A1R confocal diode lasers, Japan).

2.10 DNA Transfection Test in a Three-Dimensional (3D) Multicellular Spheroid Model.

HEK 293 3D multicellular spheroids were formed by the hanging drop method.²⁵ HEK 293 spheroids were treated with culture medium (control), Lipo2000, and DNA polyplex (N/N/P ratio of NLS/polymer/DNA at 0.25:1:20). After 48 h, HEK 293 spheroids were gently rinsed with PBS and were stained using Hoechst 33342. Samples were then imaged using the Nikon CLSM described above.

2.11 mRNA Polyplex Transfection Test.

mRNA transfection efficiency was tested in HEK 293 and RAW 264.7 cells. GFP-encoding mRNA (OZ Biosciences Inc. San Diego, CA) was used for a proof-of-concept study. Cells were seeded in a 96-well plate (Falcon, Corning, Tewksbury, MA) at a cell density of 20 000 cells per well and cultured overnight before use. Cells were then treated with culture medium as a negative control, Lipo2000/mRNA as a positive control, and mRNA polyplex (N/P ratio at 20). The dosage of mRNA was 200 ng per well. HEK 293 cells were harvested at 4, 6, 10, 24, and 48 h with 2.5% EDTA–trypsin, whereas RAW 264.7 cells were harvested through repeatedly pipetting. Cells were then spun down and resuspended in 500 μ L PBS for flow cytometric analyses. Data were analyzed using FlowJo 7.6. The transfection efficiencies of the mRNA polyplexes were determined by the percentage of GFP positive cells in the cells treated with mRNA polyplexes or Lipo2000/mRNA. Cells treated with pure media were used to define the gate for GFP negative cells.

2.12 Delivery Efficiency Test for RNP Polyplexes.

mCherry-HEK 293 cells were seeded in 96-well plate (15 000/well) and cultured overnight. Cells were then treated with culture medium (control), Lipo2000-RNP (0.75 μ L of Lipo2000 per well), and RNP polyplex at different polymer/RNP weight ratios (i.e., 10, 20, and 30). Two days later, another 100 μ L fresh culture medium was added to each well. Thereafter, half of the cell medium was replaced with an equal amount of fresh culture medium every day. At day 6, cells were digested with 0.25% EDTA–trypsin, spun down, and resuspended with 500 μ L PBS for flow cytometric analyses. Data were analyzed using FlowJo 7.6. The loss of mCherry fluorescence measured through flow cytometry was used to assay the editing efficiency.

2.13 Cell Viability Tests for DNA, mRNA, and RNP Polyplexes.

Cells were seeded in a 96-well plate (20 000/well) and cultured overnight before use. Cells were treated with pure media (control), Lipo2000, and DNA polyplex (or mRNA polyplex or RNP polyplex) for 48 h. Cell viabilities (relative to the control group) were measured with a standard MTT assay (Thermo Fisher, Fitchburg, WI). Data were collected using a GloMax-Multi Microplate Multimode Reader (Promega, Madison, WI). The concentrations of the polyplexes and Lipo2000 used for the MTT assay were the same as those used for the transfection efficiency studies. All experiments were carried out in quintuplicate.

3. RESULTS AND DISCUSSION

3.1 Polymer Synthesis and Characterization.

GSH-responsive cationic block copolymer P(Asp-AED-ICA)-PEG was synthesized, as illustrated in Scheme 1. PBLA-PEG was first synthesized by the ring-opening polymerization of BLANCA using NH_2 -PEG as the macroinitiator. It then underwent aminolysis using 2-aminoethyl disulfide (AED) to form the water-soluble cationic block polymer P(Asp-AED)-PEG. Imidazole groups were then selectively conjugated to P(Asp-AED)-PEG through an amidization reaction to enhance the endosomal escape capability of the resulting polyplexes via a proton sponge effect. The chemical structures of all polymers were well-characterized by ^1H NMR (Figure S1) and GPC (Table S1). The resulting water-soluble cationic block copolymer P(Asp-AED-ICA)-PEG can form polyplexes with negatively charged genetic biomacromolecules through electrostatic interactions.

3.2 GSH-Responsive Cationic Polymers for Nucleic Acid Delivery.

We first evaluated the potential of the GSH-responsive cationic polymer P(Asp-AED-ICA)-PEG for DNA delivery. P(Asp-AED-ICA)-PEG and DNA can readily form DNA polyplexes in an aqueous solution. The complexation was studied using an agarose gel electrophoresis assay. As shown in Figure 2A, DNA polyplexes with various N/P ratios were tested and the DNA completely lost its mobility in the electric field at an N/P ratio of 10 or above. One critical step for DNA delivery is the translocation of DNA into the nuclei to function.^{32,33} The positively charged NLS peptide, as a means to facilitate nuclear import,^{29,30} was bonded with DNA through electrostatic interactions before polymer complexation. First, the ratio of DNA/NLS/polymer was optimized in HEK cells. EGFP-encoding pDNA was used as a model pDNA. The transfection efficiency can be easily studied by quantitative flow cytometric analyses. As shown in Figure 2B, although DNA can be fully complexed at an N/P ratio of 10, better transfection efficiency was found at higher N/P ratios (i.e., 20 and 30). More importantly, the integration of a small amount of NLS peptide (N/P = 0.25) significantly enhanced transfection efficiency, whereas no difference was observed with increased amounts of NLS. The mean fluorescence intensity data (Figure 2C) showed a similar trend. The intracellular pathways of the DNA polyplexes (an N/N/P ratio of polymer/NLS/DNA at 20:0.25:1, for example) were also monitored. As shown in Figure 3, after 30 min of incubation, DNA polyplexes colocalized well with endosomes/lysosomes, indicating that they were endocytosed and trapped in the endosomes/lysosomes. At 2 h post-incubation, the extent of colocalization between the DNA polyplexes and endosomes/lysosomes significantly decreased suggesting that the majority of DNA polyplexes successfully escaped from the endosomes/lysosomes. Moreover, DNA polyplexes with NLS led to a much higher level of nucleus translocation at 6 h compared to the one without NLS. This was consistent with the higher transfection efficiency associated with NLS-attached DNA polyplexes. In summary, because the N/N/P ratios of polymer/NLS/DNA at 20:0.25:1 and 30:0.25:1 performed similarly well, we chose to use the N/N/P ratio of polymer/NLS/DNA at 20:0.25:1 in the following DNA transfection assays owing to the lesser amount of polymer required. The average hydrodynamic diameter of DNA polyplexes at this condition was 175.9 nm (PDI = 0.109; Figure S2(A)), as measured by DLS. The zeta potential of the DNP polyplexes was +4.7 mV. The stability of the DNA polyplexes was

tested in the culture media. The size changes over time were recorded by DLS. As shown in Figure S3, the sizes of the DNA polyplexes remained unchanged in FBS-containing cell culture media within 24 h, indicating good stability in cell culture media.

The integration of disulfide bonds in the polyplex system is expected to facilitate DNA decomplexation from the polyplexes once inside the cytosol that contains a high concentration of GSH (2–10 mM). Although the disulfide bond is believed to be stable in extracellular spaces where the GSH concentration is significantly lower (0.001–0.02 mM), it still raises a stability concern for our polyplexes because the degradability of disulfide bonds in different polymers may vary.³⁴ Hence, to determine at which GSH concentration the polyplexes remain stable and functional, different concentrations of GSH were intentionally added to the cell culture media during the experiments. As shown in Figure 4, the transfection efficiency did not change at a GSH concentration of 0.1 mM or lower. This indicates that the polyplexes were at least stable and functional at 0.1 mM of GSH, which is much higher than the GSH concentration in the extracellular spaces. However, at a GSH concentration of 1 mM or higher, GFP positive cells were barely observed, demonstrating that polyplexes were already disrupted before cell internalization, thus resulting in limited transfection efficiencies. These data suggest that this DNA polyplex system can remain chemically stable at extracellular GSH concentrations and can be degraded to release DNA once inside the cytosol.

We then further evaluated the potential of the DNA polyplexes in other cell lines, including HCT 116 cells, U87-MG cells, and MZ-CRC-1 cells, and compared the transfection efficiency with that of Lipofectamine 2000 (i.e., Lipo2000), a commonly used agent for in vitro gene delivery. As shown in Figure 5A, DNA polyplexes induced significantly higher transfection efficiencies than Lipo2000 in HCT 116 cells, U87-MG cells, and MZ-CRC-1 cells, whereas similar efficiencies were found in HEK 293 cells. Mean fluorescence intensity analyses, shown in Figure 5B, also confirmed this observation. The histograms of the mean GFP fluorescence intensity are shown in Figure S4. Moreover, it is well-known that Lipo2000 has a severe cell toxicity issue, which is also consistent with our results shown in Figure 5C. However, no significant cell death was observed with regard to the DNA polyplexes, suggesting that this polyplex is a safer nanoplatform for DNA delivery. DNA polyplexes were also tested in a 3D multicellular spheroid model. As presented in Figure S5, a significant number of cells were transfected.

The suitability of these GSH-responsive cationic polymers for mRNA delivery was also investigated. The average hydrodynamic size and zeta potential of the mRNA polyplex (N/P = 20) was 162.7 nm (PDI = 0.147) and +3.2 mV, respectively (Figure S2B). The GFP-encoding mRNA polyplex was tested in HEK 293 and RAW 264.7 cells at various time points (i.e., 4, 6, 10, 24, and 48 h). As shown in Figure 6A,B, in both cell lines, GFP expression was detected as early as 4 h and reached a maximum level at 24 h. Although the transfection efficiency between the mRNA polyplex treatment group and the Lipo2000 treatment group exhibited no difference at all time points, there was much less cell loss in the mRNA polyplex treatment group (i.e., <8%, Figure 6C), making it a superior nanoplatform for mRNA delivery.

3.3 GSH-Responsive Cationic Polymers for Cas9/sgRNA RNP Delivery.

Because the GSH-responsive cationic block copolymer can deliver negatively charged nucleic acids efficiently, we hypothesized that it could also deliver other negatively charged biomacromolecules, such as Cas9/sgRNA RNP³⁵ for gene editing. To verify this hypothesis, the RNP polyplexes were prepared at different weight ratios of the cationic polymer and RNP in an aqueous solution. As mentioned earlier, RNP functions in the cell nucleus. Hence, to promote nuclear transportation, Cas9 protein modified with NLS (sNLS-Cas9-sNLS) was used in this study. The hydrodynamic diameter of the resulting RNP polyplexes (weight ratio of polymer/RNP at 20, for example) was 160.8 nm (PDI = 0.155; Figure S2C). For a proof-of-concept study, the functionality of the RNP polyplex system was tested in an mCherry-expressing HEK 293 cell line. The RNP used here can target and knockout mCherry transgenes,³⁶ thus inducing the loss of mCherry fluorescence that can be easily detected by flow cytometry. As shown in Figure 7A, a significant reduction of mCherry fluorescence intensity compared to the untreated control group indicates that RNP had been successfully delivered via RNP polyplexes. Among all weight ratios, the RNP polyplex at a weight ratio of 20 exhibited the highest gene editing level (~51%), which was slightly higher than that of Lipo2000, a state-of-the-art commercially available RNP transfection reagent. However, as demonstrated above, the polyplex system exhibited much better biocompatibility than Lipo2000 (Figure 7B). Beyond that the RNP polyplexes can be conveniently modified with functional moieties (e.g., targeting ligands and imaging probes), enabling them to outperform Lipo2000.

4 CONCLUSIONS

In this study, a universal GSH-responsive nanoplatfrom capable of delivering negatively charged genetic biomacromolecules (i.e., DNA, mRNA, and Cas9 RNP) for gene therapy was developed. The polyplexes were easily formed in an aqueous solution by electrostatic interactions between anionic genetic payloads and cationic P(Asp-AED-ICA)-PEG block copolymers bearing imidazole residues and disulfide bonds. The study on the chemical stability of the GSH-responsive polyplexes revealed that the polyplexes were able to remain stable at extracellular GSH concentrations, while being readily degraded to release their payloads at intracellular GSH concentrations. Moreover, the polyplexes showed great transfection efficiency and good biocompatibility in multiple cell types and out-performed the commonly used cationic lipid-based delivery system, Lipo2000. Furthermore, the ease of surface modification of the polyplex system enables its broader applications both in vitro and in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic depiction of the proposed intracellular pathways of the DNA polyplex, mRNA polyplex, and Cas9 RNP polyplex. Polyplexes were formed through electrostatic interactions. After the polyplexes were taken up by cells through endocytosis, they were able to escape effectively from the endosomes through a proton sponge effect owing to the presence of imidazole groups. Once in the cytosol, GSH cleaved the pendent –S–S– bonds in the polymers, thereby converting the cationic polymers to neutral polymers and thus releasing the payloads. The NLS peptides facilitate nuclear import of DNA and Cas9 RNP.

Figure 2.

(A) Agarose gel electrophoresis of DNA polyplexes at various N/P ratios. (B) Transfection efficiency and (C) mean fluorescence intensity of various DNA polyplex formulations tested in HEK 293 cells. Transfection was carried out at a dose of 250 ng of EGFP pDNA per well in a 96-well plate. The intensity of GFP fluorescence was measured directly by flow cytometry. The experimental data are represented as mean $\pm$ standard error (SE, $n = 3$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Figure 3.

Intracellular trafficking of DNA polyplexes with and without NLS (N/N/P ratio of polymer/NLS/DNA at 20:0.25:1). Scale bar: 10 μ m.

Figure 4. Transfection of DNP polyplexes (N/N/P ratio of polymer/NLS/DNA at 20:0.25:1) in GSH-containing media. The experimental data are shown as mean $\pm$ SE ($n = 3$). n.d.: not detectable.

Figure 5.

(A) Transfection efficiency, (B) mean fluorescence intensity, and (C) cell viability of DNA polyplexes (N/N/P ratio of polymer/NLS/DNA at 20:0.25:1) in HCT 116, U87-MG, MZ-CRC-1, and HEK 293 cells. The experimental data are represented as mean $\pm$ SE ($n = 3$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Figure 6.

(A) Transfection efficiency, (B) mean fluorescence intensity, and (C) cell viability of mRNA polyplexes (N/P ratio = 20) in HEK 293 and RAW 294.7 cells. The experimental data are represented as mean ± SE ($n = 3$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Figure 7.

(A) Optimization of the RNP polyplexes in vitro using mCherry-expressing HEK 293 cells. mCherry-HEK 293 cells were treated with RNP polyplexes with different weight ratios of polymer/RNP, and the loss of mCherry fluorescence was measured through flow cytometry to evaluate the genome editing efficiency. (B) Cell viability of RNP polyplexes (weight ratio = 20). * $p < 0.05$; ** $p < 0.01$.

Scheme 1.
Synthetic Scheme of the Cationic Block Copolymer P(Asp-AED-ICA)-PEG