A zwitterionic polymer-inspired material mediated efficient CRISPR-Cas9 gene editing

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\section*{A R T I C L E   I N F O}

Article history:
Received 16 April 2022
Revised 8 July 2022
Accepted 22 August 2022
Available online 20 September 2022

Keywords:
CRISPR/Cas9
Gene editing
Zwitterionic polymers
CD44
PLK1

\section*{A B S T R A C T}

The type II prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR/Cas9) adaptive immune system is a cutting-edge genome-editing toolbox. However, its applications are still limited by its inefficient transduction. Herein, we present a novel gene vector, the zwitterionic polymer-inspired material with branched structure (ZEBRA) for efficient CRISPR/Cas9 delivery. Polo-like kinase 1 (PLK1) acts as a master regulator of mitosis and overexpresses in multiple tumor cells. The Cas9 and single guide sgRNA (sgRNA)-encoded plasmid was transduced to knockout PLK1 gene, which was expected to inhibit the expression of PLK1. Our studies demonstrated that ZEBRA enabled to transduce the CRISPR/Cas9 system with large size into the cells efficiently. The transduction with ZEBRA was cell line dependent, which showed ~10-fold higher in CD44-positive cancer cell lines compared with CD44-negative ones. Furthermore, ZEBRA induced high-level expression of Cas9 proteins by the delivery of CRISPR/Cas9 and efficient gene editing of PLK1 gene, and inhibited the tumor cell growth significantly. This zwitterionic polymer-inspired material is an effective and targeted gene delivery vector and further studies are required to explore its potential in gene delivery applications.

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Peer review under responsibility of Shenyang Pharmaceutical University.

https://doi.org/10.1016/j.ajps.2022.08.001
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1. Introduction

The type II prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR/Cas9) adaptive immune system is a cutting-edge genome-editing toolbox to induce point mutations, gene deletions/insertions, chromosomal translocations, and many other DNA manipulations [1,2]. Given the powerful capability of the CRISPR/Cas9 system in gene editing, many challenging disorders, such as cancers [3], retinoblastoma [4], tyrosinemia [5], and hyperlipidemia [6], may be cured of the genetic level. However, the therapeutic effects of CRISPR/Cas9 are impeded by low delivery efficiency [7]. Viral vectors, the traditional gene delivery measure, are limited in their applications due to their inflammatory, immunogenic properties, and oncogenic insertional mutagenesis [8]. Addressing these issues, non-viral nanoparticles for delivery of the CRISPR/Cas9 system in formats of plasmid, mRNA, and Cas9/sgRNA ribonucleoproteins (RNPs) have been reported. Cell-penetrating peptides [9], DNA nanoclewls [10], cationic polymers [11], and lipids [12], were promising as non-viral vectors. However, these vectors showed limitations in the in vivo transfection. We developed lipid-based nanoparticles for successful in vivo delivery of CRISPR/Cas9 and tumor therapy [13]. However, the in vivo applications of non-viral vectors are still challenged by the relatively low accuracy. We thus present a light-activated lipid-gold nanosystem that realized the spatiotemporal release of CRISPR/Cas9 for accurate tumor therapy [14]. To realize accurate therapy, other stimuli-responsive nanocarriers such as a pH-responsive vehicle [15], redox-responsive delivery systems [16], reducible delivery systems [17,18], in situ self-assembly vehicles [19], and nanomagnetic system [20], were also developed. Nevertheless, the above non-specific targeting methods still face problems such as inapplicable for disorders in deep organs and the high systematic dosage. To overcome the disadvantages, we employed an active targeting strategy for specific targeting liver cells and realized efficient regulation of hyperlipidemia. Moreover, this strategy displayed a high level of biosafety [6]. Thus, the tissue-specific delivery is promising for CRISPR/Cas9 to optimize its effectiveness and simultaneously minimize its side effects [19].

Zwitterionic polymers have emerged as a novel class of attracting materials, for they show superior performance in “smart” adaptiveness and nonspecific adsorption [21]. Zwitterionic polymers consist of oppositely charged cationic and anionic groups along the chain or side chain, which are different from the true polyelectrolytes (polyanions or polycations) with the same charge of monomers in the polymer [22]. This combination of oppositely charged groups in the polymers grants them typically stimuli-responsiveness and dual-nature properties. The performance of zwitterionic polymers switches between antipolyelectrolyte and polyelectrolyte, which depends on their environment. Furthermore, this kind of polymer possesses superior hydrophilicity, which effectively resists the nonspecific adsorption or “fouling” from biomolecules [23]. Previous reports indicated that the subcutaneous implantation of zwitterionic poly(carboxybetaine methacrylate) hydrogels in the mice showed resistance to form fibrotic capsules for at least 3 months [24]. The zwitterionic coating composed of poly(sulfobetaine methacrylate) and polydopamine enabled to suppression of the acute inflammatory brain tissue response to implants effectively [25]. However, the use of zwitterionic polymers as gene delivery systems showed some disadvantages, such as the neutral charge reducing the attraction of negative gene medicines and the protection from enzyme degradation. There is great significance to developing a novel style of materials possessing both functions of zwitterionic polymer and the efficient capability to carry gene medicines.

Herein, we developed a zwitterionic polymer-inspired material with a branched structure (ZEBRA), a ternary copolymer with high transfection efficiency, specific targeting capability, and high biosafety for CRISPR/Cas9 delivery. The ternary copolymer was composed of agarose (AG), low molecular weight polyethyleneimine (LMW PEI), and hyaluronic acid (HA), which were expected to function synergistically. HA is a primary binding molecule targeting CD44, which is over-expressed in a variety of solid tumors, such as pancreatic, breast, and lung cancer [26]. The HA block on the ZEBRA was designed to realize preferential tumor accumulation and increased cell uptake. The LMW PEI block can provide necessary cationic characteristics for cell membrane penetration and CRISPR/Cas9 adsorption via electrostatic interactions, and lysosome escape due to the proton-sponge effect caused by the high density of ionizable amines. The gelling property of the AG block was designed to promote gene compaction. Also, the abundant hydroxyl in the AG block may provide general attachment sites for conjugating PEI. Thus, the ZEBRA can encapsulate and condense CRISPR/Cas9, target tumor cells, and deliver CRISPR/Cas9 into the cells. We selected the polo-like kinase 1 (Plk1) gene, a master regulator of mitosis overexpressed in tumor cells, as the targeting site of CRISPR/Cas9 to evaluate the efficacy of the ZEBRA in delivering pCP (plasmid-encoded with Cas9-sgPlk1) for tumor therapy.

2. Materials and methods

2.1. Materials

The sodium salt of hyaluronic acid (Mw ~5 kDa, Purity 99%) was purchased from Freda Biopharm Co., Ltd. (China). Agarose (AG, Mw ~3 kDa) was prepared following an H2O2 degradation protocol [27]. PEI with Mw of 1.2 kDa (Purity 99%) and 25 kDa (Purity 99%), N-(3-dimethylpropyl)-N-ethylcarbodiimide hydrochloride (EDC, Purity ≥99%), N-hydroxysulfo-succinimide sodium salt (sulfo-NHS, Purity 98%), and 1, 1-Carboxybiimidazole (CDI, Purity 97%) were obtained from Sigma-Aldrich (USA). FX458 plasmid was obtained from Addgene (Cambridge, USA). All other commercial reagents are analytical grade and were used without further purification.

2.2. Synthesis of AG grafting PEI (AG-PEI)

AG-PEI was synthesized by the conjugation of hydroxyl and primary amino. Briefly, AG (0.56 g, 0.18 mmol) and CDI (0.5 g,
3 mmol) were first dissolved in anhydrous dimethyl sulfoxide (DMSO, Purity ≥99.9%). The mixture was stirred at room temperature for 1.5 h and then precipitated in cold ethanol. The resulting AG-CDI was filtered, dissolved in DMSO, and stored at 4 °C. To obtain AG-PEI, PEI 1.2 kDa (1.80 g, 1.5 mmol) was dissolved in DMSO, and then the AG-CDI in DMSO and triethylamine were slowly added dropwise to the PEI solution over 1.5 h with stirring, and the mixture was allowed to react for 5 h. After the reaction, the mixture was dialyzed in water for 10 d using dialysis tubing (MWCO, 2 kDa). The resulting AG-PEI aqueous solution was concentrated by rotary evaporation (IKA RV10 rotary evaporator, Germany) and the AG-PEI was lyophilized to obtain the purified AG-PEI.

2.3. Synthesis of ZEBRA

The reaction was carried out according to the instructions for EDC. Briefly, the pH value of 10% (w/v) AG-PEI solution (10 ml) was adjusted to about 7, followed by the addition of 1% (w/v) HA solution (10 ml). Then 30 mM EDC and 8 mM N-Hydroxysuccinimide were added under nitrogen atmosphere, and the reaction was carried out under nitrogen atmosphere for 5 h with stirring. The product (ZEBRA) was obtained after purification by dialysis in water for 5 d with dialysis tubing (MWCO, 5 kDa) and finally, the purified ZEBRA was collected.

2.4. Characterization of ZEBRA

Samples were mixed with KBr and compressed into disks, and their FT-IR spectra were recorded by an FTIR spectrometer (Spectrum 2000, Perkin Elmer). The Carbon-13 nuclear magnetic resonance (13C NMR) spectra of the samples were dissolved in D2O/CF3COOD (6/1, v/v) and recorded on a Bruker AVANCE III –500 MHz NMR spectrometer at 500 MHz at room temperature. ZEBRA was dried under vacuum, and their elemental analyses were performed on a PerkinElmer CHNS/O 2400 elementary analyzer.

2.5. Degradation of ZEBRA in simulating cellular environments

ZEBRA was incubated with 0.25% trypsin in PBS buffer at pH 7.2 or 0.5 mg/ml pepsin in acetate/sodium acetate buffer at pH 5.0 at 37 °C on a rotary shaker, respectively. Meanwhile, ZEBRA incubated in PBS buffer at pH 7.2 or acetate/ sodium acetate buffer at pH 5.0 without any enzyme was used as control. After the incubation for 48 h, the reaction mixture was heated at 80 °C for 10 min to inactivate the enzyme, subsequently ultra-filtered. The molecular weight of degradation products under different conditions was determined by Gel permeation chromatography (GPC) (515–410 Waters, USA).

2.6. Preparation and characterization of ZEBRA-based polyplexes

The plasmid encoded with green fluorescent protein (GFP) sequence was constructed (pGFP). The polyplexes prepared at various N/P ratios (ranged from 1 to 100) with the PEI 1.2 kDa, AG-PEI, or ZEBRA were produced via self-assembly in 150 mM PBS buffer (pH 7.4) by mixing pGFP, and then vortex for 30 s, respectively. The polyplexes were kept at room temperature for 30 min before use. The volumes of the solutions were adjusted to 500 μl with Opti-MEM Reduced-Serum Medium for transfection.

The particle size and zeta potential of the polyplexes were measured using a Zetasizer Nano-ZS dynamic (Malvern Instruments, USA). The polyplexes were prepared in PBS buffer (pH 7.4) at various N/P ratios. The morphology of polyplexes was characterized under a Philips Tecnai 10 TEM.

2.7. Serum nuclease protection assay

To evaluate the ability of ZEBRA of protecting the condensed plasmids from serum nuclease, the pGFP or ZEBRAG prepared at the N/P ratio of 10 was incubated at 37 °C with 10% FBS containing solutions and withdrawn at different time intervals (0, 0.5, 1, 2, 3 and 6 h), followed by incubating with 5 μl of 0.1 M EDTA solution for 10 min to inactivate serum nuclease. And then, 10 μl heparin (5 mg/ml) was added and incubated for 2 h to completely dissociate polyplexes. The analysis of plasmids degradation was performed by gel electrophoresis.

2.8. Protein absorption

The blood was obtained from BALB/c nude mice. The plasma was collected and stored at −80 °C. Different formulations, such as PEI 1.2 kDa/pGFP, PEI25kDa/pGFP, and ZEBRAG, were prepared at the N/P ratios of 1, 5, 10, 20, 50 and 100, respectively. The dispersion was incubated with 400 μl plasma for 1 h at 37 °C with stirring. The nanoparticles were removed from the supernatant by centrifugation at 20 000 g for 15 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to analyze the protein compositions.

2.9. Stability of the nanoparticles

ZEBRAG, PEI 25 kDa/pGFP, and AG-PEI/pGFP were prepared at a N/P ratio of 10. The nanoparticles were dispersed in PBS. DLS was used to analyze the size of the nanoparticles within 3 d. Furthermore, ZEBRAG was prepared at a N/P ratio of 10, and incubated in DMEM medium with no serum, Opti-MEM medium, and DMEM medium supplemented with 10% FBS. DLS was used to analyze the size of the nanoparticles.

2.10. In vitro toxicity

The HeLa cells, human umbilical vein endothelial cells (HUVECs), and normal human liver cells (HL-7702) were seeded with a density of 5 × 10^3 cells per well (Corning, USA) for 24 h before the experiment. Subsequently, the culture medium was replaced with 200 μl polyplexes with varied N/P ratios (1–100) with the dosage of pGFP equivalent to 2 μg/ml. The cells were incubated for another 24 h. Each concentration was replicated in 5 wells. Thereafter, 20 μl CCK-8 (Dojindo, Japan) was added to each well and the cells were incubated for another 2 h. Then, the optical density readings were performed using an Ultra Multifunctional Microplate Reader (Tecan, USA) at a wavelength of 450 nm. The absorbance was read relative to the blank well. Cell viability (%) in each well
was calculated by the formulation:

\[
\text{Cell viability} \% = \frac{(OD_{450 \text{rest}} - OD_{450 \text{blank}})}{(OD_{450 \text{control}} - OD_{450 \text{blank}})} \times 100\%.
\]

Where, \(OD_{450 \text{rest}}\): the OD value of the formulation treated cells; \(OD_{450 \text{control}}\): the OD value of the non-treated cells; \(OD_{450 \text{blank}}\): the OD value of the medium.

To study the polyplex interactions with blood components, the polyplexes were incubated with citrated mouse blood for 30 min at 37 °C. The supernatant was collected by centrifugation at 4000 rpm for 5 min. The resulting supernatant was subjected to 10% SDS-PAGE and separated with a Mini-PROTEAN II electrophoresis cell (Bio-Rad, USA) at 100 V for 90 min, followed by Coomassie blue staining. The image of the gel was taken using a LAS4000 image analyzer. The sample consisting of blood and normal saline was used as the control.

We also evaluated the hemolysis induced by the nanoparticles. The fresh mouse blood was collected in the presence of sodium citrate, followed by centrifugation at 5000 rpm for 5 min. We collected the red blood cells (RBCs) after the cell pellets was washed thrice. A volume of 0.5 ml RBCs (2.0%, v/v) was mixed with 0.5 ml ZERBAG (pGFp equivalent to 2 µg/ml). The N/P ratios were 0, 1, 5, 10, 20, 50 and 100, respectively. The RBC suspension was mixed with PBS or 1.0% Triton X-100 solution was used as a negative or positive control, respectively. The mixtures were incubated at 37 °C for 1 h and centrifuged at 1 x \(10^4\) rpm for 10 min. We tested the absorbance of the supernatant by an Ultra Multifunctional Microplate Reader (Tecan, USA) at 540 nm (belonging to hemoglobin). We calculated the percentage of hemolysis as follows:

\[
\text{Hemolysis} \% = \frac{[(A_{t} - A_{n})/(A_{p} - A_{n})]}{100}\%.
\]

### 2.11. In vitro gene transfection studies

HeLa cells with a density of \(1 \times 10^5\) cells/ml were seeded into 24-well plates, and cultured at 37 °C for 24 h in DMEM medium supplemented with 1% Penicillin/Streptomycin and 10% FBS in a humidified atmosphere with 5% CO₂. To screen the most efficient sgRNA, different sgRNA sequences were cloned into the PX458 plasmid. HeLa cells were transfected with ZEBRA complexed plasmids encoded with four different sgRNAs (pCP-1, pCP-2, pCP-3, and pCP-4), respectively. The plasmids were performed at a weight of 2 µg. To evaluate the gene-editing efficiency, HeLa cells with a density of \(3 \times 10^5\) cells/ml were seeded into 6-well plates and incubated for 24 h. We replaced the medium with OptimEM serum reduced medium containing PBS, ZEBRA, CP, ZEBRA/Cas9-sgLuciferase (ZEBRAL), Lipo2000/pCP (LCP), or ZEBRAPP treatment, respectively. After the cells were incubated with different formulations for 4 h, we replaced the medium with a freshly prepared DMEM complete medium and further incubated the cells for 48 h. The cells were washed with PBS thrice, trypsinized, and harvested. The DNA genomes were extracted with a QuickExtract DNA Extraction Kit (Epicentre Biotechnologies, USA). The targeting DNA fragments at the Plk1 loci were amplified by Polymerase Chain Reaction (PCR) with the primers as below. T7 Endonuclease I assay was performed according to our previous work [6]. The mutation frequency was calculated according to the formula as below.

\[
F_m \% = \frac{[B_2 + B_3]/[B_1 + B_2 + B_3]}{100}\%.
\]

Where, \(B_1\) is the integrated intensity of the undigested PCR product; \(B_2\) and \(B_3\) are the integrated intensities of each cleavage product.

Primers: Plk1-F: CTCAATCCACCTCCCCATCC

Plk2-R: TCTCCCTTCTTTCCTCCAGC

### 2.13. Intracellular trafficking

To study intracellular trafficking, Sulfo-Cyanine5 (Cy5) labeled pCP was prepared following the supplier’s protocol and our previous work [13]. The commercial regent Cy5 or Cyanine 7 (Cy7) (Mirus Bio, USA) was used to incubate with pCP plasmid at 37 °C for 1 h. The dye-labeled pCP (Cy5-pCP or Cy7-pCP) was purified with G50 Microspin Purification Column and washed with labeling buffer twice. We mixed the eluent with 0.1 vol of sodium chloride (5 M) and 2 vol ice-cooled 100% ethanol, followed by the placement at −20 °C for 30 min. The mixture was centrifuged at 14 000 g for 30 min and the supernatant was removed. We added the ethanol (70%) at a volume of 500 µl to the pellets, followed by the centrifugation at 14 000 g for 30 min. The pellets were resuspended with sterilized water and stored at −20 °C.

HeLa cells were treated with Cy5-labeled nanoparticles (Cy5-ZEBRAP) in the serum-free medium. After incubation with the polyplexes for a different time or incubation with the polyplexes at different concentrations, cells were washed twice with the pre-warmed PBS and then were fixed with 4% paraformaldehyde in PBS (pH 7.2) for 15 min at room temperature. FITC labeled phalloidin (Thermo Fisher Scientific, USA) was used to stain the F-actin according to (Scheme S1). The resulting plasmids were corresponding to pCP-1, pCP-2, pCP-3, and pCP-4, respectively.
the manufacturer’s protocol [28]. After washing with PBS (pH 7.4), nucleus staining was performed with DAPI solution (5 μg/mL) for 30 min at room temperature in the dark and the cells were washed with PBS repeatedly. Cells transfected with Lipofectamine™ 2000 (denoted as Lipo2000) (Invitrogen, USA) were used as the positive control, and those without any treatment were used as the negative control. The cells were examined using a confocal laser scanning microscopy (CLSM, LSM 880, CarlZeiss, Germany). For excitation of DAPI fluorescence, a wavelength of 364 nm laser was used. The excitation of Cy5 was performed with a helium-neon laser at 633 nm; an argon laser with a wavelength of 488 nm was used for FITC-phalloidin. The statistical analysis was carried out using CLSM 880 software. The uptake ratios in various culture times and mass of ZEBRA polyplexes were carried out similarly. To quantify the cellular uptake of polyplexes, cells were plated on 24-well plates and transfected with Cy5-ZEBRAP. After that, cells were trypsinized, washed with PBS twice, and suspended in 100 μl of PBS. The percentage of Cy5 positive cells was examined by flow cytometry (FACS).

Different cell lines, including MDA-MB-231, MCF-7, and NIH-3T3 cells were cultured in confocal culture dishes at a density of 10^5 cells/dish. After 24 h incubation, the DMEM medium was removed and 1 mL polyplex solutions (Cy5-pCP equivalent to 2 μg) were added to the cells and transfected for 4 h, and then the culture medium was again replaced with fresh medium. The cells were treated and examined by CLSM.

2.14. Lysosome escape

HeLa cells were seeded on confocal culture dishes at a density of 1 x 10^5 cells/mL and incubated for 24 h. The Opti-MEM reduced serum medium containing ZEBRAG was added to the cells and incubated for 1 h or 4 h at 37 °C with 5% CO₂, followed by staining with LysoTracker Blue DND-22 for 30 min. Next, the medium was removed, washed with PBS thrice, and observed under a CLSM 880. The excitation wavelength of the dyes is 365 or 633 nm, corresponding to LysoTracker Blue DND-22 or Cy5. The statistical analysis was performed by CLSM 880 software.

2.15. Antibody blocking

HeLa cells were seeded on confocal culture dishes at a density of 1 x 10^5 cells/mL and incubated for 24 h. The Anti-CD44 antibody (10 μg/mL) was incubated with HeLa cells for 30 min at 37 °C, followed by incubation with Cy5-ZEBRAP for 4 h. We analyzed the cells with CLSM and FACS. Excitation/Emission: Cy5, 640/670.

2.16. Evaluation of the specificity of CRISPR/Cas9

We evaluated the specificity of CRISPR/Cas9 by predicting the off-target sites with the CRISPR Design server (http://crispr.mit.edu/). Ten sites based on either sequence similarity (three-base mismatches) or high scoring (the top-scoring four-base mismatches) were selected (Table S1). We found no site that was zero-base, one-base, or three-base mismatch. The targeting DNA fragments at the ten loci were amplified by PCR with the primers in Table S2. T7EI assay was performed as mentioned above.

2.17. Western blotting (WB) assay

The expression of specific genes was characterized by WB assay. For the in vitro experiment, the transfected cells were rinsed twice with PBS, followed by the treatment with a lysis buffer (10% glycerol; 150 mM NaCl; 0.2% Triton100; 1 mM DTT; 50 mM Tris, pH 8; protease inhibitor cocktail, Roche; phosphatase inhibitor cocktail I, AG Scientific and II, Calbiochem). The proteins were resolved by 10% SDS-PAGE and transferred onto PVDF membranes, followed by blocking with milk for 1 h at room temperature. After the incubation with anti-PLK1 mouse monoclonal (Abcam, USA), the membrane was washed and incubated with goat anti-mouse secondary antibody and scanned with an Amersham Imager 600 system (GE, USA). For the tissues, the Western blot analysis was performed after the tumor lysates were obtained, and probed with antibodies against PLK1 and GAPDH (Abcam, USA).

2.18. Animals

All animals were from Vital River Laboratory Animal Center (Beijing, China). The animals were raised in a specific pathogen-free (SPF) environment. All in vivo studies were performed following the Institutional Authority for Laboratory Animal Care of Guangzhou Medical University (GY2022–046).

2.19. Photoacoustic (PA) imaging

To evaluate the specificity in vivo, a PA system (Vevo LAZR-X, Fujifilm, Japan) was used to characterize the accumulations of ZEBRAP in the HeLa tumor-bearing mice. Cy7-labeled plasmids were used to prepare different formulations, including Cy7-pCP, AG-PEI/Cy7-pCP, and Cy7-ZEBRAP. The mice were administrated with these formulations in a volume of 200 μl via intravenous injection in the tail vein. For PA analysis, we detected the PA signals in the tumors at various time points (1, 6, 12, 24, and 48 h). Before the detection, the mice were anesthetized with 5% isoflurane. PA signals were presented as the subtraction of background signals.

2.20. The tumor inhibition assay

BALB/c nude mice bearing HeLa tumors were injected intratumorally with Saline, ZEBRA, pCP, ZEBRAL (ZEBRA/Cas9-sgluciferase), Lipo2000/Cp (LCP), or ZEBRAP every 3 d (n = 5). After the post-injection of polyplexes, the mice were sacrificed with carbon dioxide. The tissues including heart, liver, spleen, lung, kidney, and tumors were harvested and then stained with hematoxylin and eosin. Histological and histomorphometric observations were performed using a light microscope (Olympus, Japan).

2.21. Statistical analysis

All experimental data were expressed as mean ± SD. Statistical analysis was performed by Student’s unpaired t-
test between two groups or One-Way Analysis of Variance (ANOVA) followed by Tukey’s posthoc test among three or more groups. The difference was considered statistically significant when P value was less than 0.05.

3. Results and discussion

3.1. The preparation of ZEBRA

We performed two-step reactions to synthesize the ZEBRA with AG, LMW PEI, and HA (Scheme 1 and Fig. S1). The $^{13}$C NMR spectra were used to analyze ZEBRA (Fig. 1A). The peak at 60.69 ppm in the AG spectrum was assigned to the carbon resonance of C$_6$ [29]. In the AG-PEI spectrum, the chemical shift values between about 35 ppm and 56 ppm corresponding to the carbon resonances of PEI, and their magnifications also showed in the rectangle. A new peak appearing at about 61 ppm (blue oval) indicated reaction happened to C$_6$ of AG; and the peak at 165 ppm belonged to carboxylic groups [30], for the coupling agent reacted with a primary amine and formed the new group. In the ZEBRA spectrum, the resonance peaks from both HA and AP demonstrated the occurrence of the grafting reaction. The peak at 16.31 ppm was assigned to the acetamido moiety of the N-acetyl-d-glucosamine residue of HA, while the peaks between 165 and 175 ppm referred to the congregation of -CONH- and -COO- groups [31].

FTIR analysis (Fig. 1B) indicated that AG was conjugated with PEI 1.2 kDa, three characteristic absorption bands belonging to PEI at 1653 cm$^{-1}$ (amide I, C = O stretching) [32], 1546 cm$^{-1}$ (amide II, N–H bending), and 1290 cm$^{-1}$ (amide III, N–H bending) [33], respectively, appeared in the spectrum of ZEBRA. The stretch vibration characteristic peaks of C–H assigned to methylene of PEI appeared at 2936 and 2848 cm$^{-1}$ [34]. The IR spectrum of ZEBRA showed higher intensity in the amide I and lower intensity in N–H stretching bands, suggesting the increase of amide groups. The methyl peak of HA at about 2979 cm$^{-1}$ covered the peak belonging to methylene of PEI after the conjugation of HA [35]. The expanded vibration absorption peak around 3400 cm$^{-1}$ was attributed to the stretching vibration of amide and hydroxyl in HA. These results suggested that ZEBRA was successfully synthesized.

Elemental analysis (Table S3) showed that the nitrogen contents of AG-PEI and ZEBRA were 14.89% and 13.13%, respectively. Because the nitrogen contents of pure PEI and HA are 19.57% and 3.51% respectively, we could determine that the weight ratio of AG to PEI 1.2 kDa in AG-PEI was approximately 1:3 and the weight ratio of AG-PEI to HA was approximately 5.5:1 in ZEBRA. Based on these calculations, the average AG molecule was grafted with ~7.5 PEI molecules and 2.3 HA molecules.

As expected, the in vitro degradation assay showed that ZEBRA degraded in the acidic (pH 5.0) or enzymatic environment (Fig. 1C), resulting in degrading ZEBRA into LMW ones. The feature of biodegradability may facilitate the efficient release of CRISPR/Cas9 and a short retention time of ZEBRA in the body.

3.2. The characterization of ZEBRA-based polyplexes

We evaluated the capability of ZEBRA in carrying nucleic acids by using a green fluorescent protein (GFP) sequence encoded plasmid (pGFP, ∼10 000 bp). The formulated polyplexes of ZEBRA and pGFP were named as ZEBRAG. The Zeta potential of ZEBRAG remained lower than +10 mV at various N/P ratios (1, 5, 10, 20, 50 and 100) (Fig. S2). ZEBRA showed an entrapment efficiency of over 80% with an N/P ratio higher than 10 (Fig. S3). Correspondingly, the loading efficiency of ZEBRAG was lower than 20% with the N/P ratio higher than 10 (Fig. S3). To ensure the biocompatibility of the nanocarriers, the toxicity of PEI 1.2 kDa/pGFP, PEI 25 kDa/pGFP, and ZEBRAG was tested. The cytotoxicity assay on HeLa cells showed that more than 80% of the cells were still viable after being treated with PEI 1.2 kDa/pGFP or ZEBRAG within the N/P ratio of 50 (Fig. S4), indicating much higher biosafety of the polymers and the polyplexes, compared with PEI 25 kDa. Furthermore, the cytotoxicity assay on normal cells such as HUVECs and HL-7702, also indicated that ZEBRAG was highly safe with the N/P ratios less than 20 (Fig. S5). The blood compatibility assay showed that PEI 1.2 kDa/pGFP or ZEBRAG had no visible effects on plasma proteins compared with the negative control (NC, PBS treatment) (Fig. S6), implying the reduced protein absorptions. In contrast, as a control, PEI 25 kDa/pGFP led to the loss of some plasma proteins (Arrows indicate the lost proteins). The quantitative analysis indicated that ZEBRAG induced significantly lower protein absorptions (Fig. S7). Moreover, ZEBRAG showed no significant hemolysis (Fig. S8 and S9). We evaluated the stability of ZEBRAG with DLS analysis. ZEBRAG showed good stability within 3 d, with the size increasing slightly in PBS (Fig. S10). The further evaluation of ZEBRAG in different medium indicated that ZEBRAG was

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**Scheme 1** – Schematic illustration of the synthesis and the transfection of ZEBRAG. (A) The formation of ZEBRAG; (B) Cellular internalization of ZEBRAG.
more stable in the medium with lower serum within 3 d (Fig. S11). But ZEBRAG maintained the size of ZEBRAG at ~200 nm for more than 24 h in the medium, which was beneficial to the effective transfection (Fig. S11). We also studied the transfection efficiency induced by ZEBRAG, which showed a large number of GFP-positive cells when the N/P ratio of ZEBRA to pGFP was larger than 10 (Fig. S12), and even better than the commercial regent Lipo2000. However, the increase of N/P ratios did not increase the transfection efficiency significantly. The control groups, such as PEI 1.2 kD or PEI 25 kD showed much lower transfection efficiency. Based on the evidence, further studies were performed with an N/P ratio of 10 ZEBAR to the plasmid.

We characterized the plasmid-containing polyplexes. TEM images showed that PEI 1.2 kD/pGFP formed aggregates with an average size of 1200 nm, demonstrating that PEI cannot effectively encapsulate the plasmid (Fig. 2A). However, the conjugation of PEI 1.2 kD to the AG (AG-PEI) compacted the plasmids into spherical particle structures with a homogeneous diameter of ~150 nm (Fig. S13), which was similar to the PEI 25 kD (Fig. 2B). The particle size of ZEBRAG was considerable to PEI 25 kD/pGFP, although the charge density was much lower compared with PEI 25 kD. This spherical and compact structure may be attributed to the high affinity between the PEI residue and the plasmids and the gelling effect of the AG chain at room-temperature (Fig. 2C). AG gel electrophoresis indicated that ZEBRA retarded the migration of pGFP significantly, implying the plasmid was bound by ZEBRA (Fig. 2D). Serum nucleases protection assay indicated that ZEBRA protected the plasmids from degradation (Fig. 2E), which provided an important shielding to protect the plasmids in the physiological environment.

These results demonstrated that ZEBRA could encapsulate large-sized plasmids to form stable polyplexes, which showed low cytotoxicity, reduced protein absorption, prevention from enzyme degradation, and efficient transfection. Thus, ZEBRA showed great potential in the delivery of CRISPR/Cas9 plasmids.

3.3. The evaluation of cellular uptake

Based on the excellent performance of ZEBRA in the delivery of reporter genes, we evaluated the delivery efficiency of pCP encoded plasmids by testing its internalization behaviors on HeLa cells. Firstly, a T7EI assay was performed to optimize the Cas9-sgPlk1 plasmid. The pCP-2 showed the most effective cutting (Fig. S14), and the plasmid was used henceforth.

To visualize the nanoparticles under CLSM, the dye Sulfo-Cyanine 5 was used to label pCP (Cy5-pCP). ZEBRAPs with an N/P ratio of 10 were performed to optimize the transfection conditions. ZEBRA/Cy5-pCP (Cy5-ZEBRAP) can enter the cells at a low concentration (Cy5-pCP equivalent to 0.5 |g/ml). The cellular uptake of Cy5-ZEBRAP increased with the elevation of the concentration, and the concentration with a Cy5-pCP equivalent to 2 |g/ml showed the most efficient cellular uptake (Fig. 3A), which was demonstrated by the strong fluorescence signals of Cy5. The quantitative analysis by FACS indicated that the Cy5-positive ratio (CPR) increased from 64.9% to 99.3% with the concentrations of Cy5-ZEBRAP increasing from 0.5 to 2 |g/ml. Significantly, the mean fluorescence intensity (MFI) increased from 2394 to 32,624, implying a sharp increase in cellular uptake. A further study revealed that the cellular uptake of Cy5-ZEBRAP increased with incubation time (Fig. 3B). The cellular uptake was significant within 1 h (CPR, 84.9%; MFI, 10,668) and reached a
The high level of CFR after 2 h (CPR, 99.2%; MFI, 21,638) (Fig. 3B). The further incubation to 4 h showed a maximum CPR and MFI (99.5%; 30 094), respectively. Thus, the optimized transfection condition was pCP concentration of 2 μg/ml and incubation for 4 h. Based on the transfection conditions, we found that free pCP, PEI1.2 kDa/pCP, PEI25kDa/pCP, and Lipo2000/pCP (LCP) showed much lower cellular uptake (Fig. S15), compared with ZEBRA. Although the high cellular uptake of pCP did not mean high gene-editing efficiency, enough plasmids increased the chance for the subsequent efficient gene-editing. This implied that ZEBRA possessed great superiority for the delivery of pCP.

Successful lysosomal escape is a critical step for efficient gene therapy [36,37]. We found that ~70% of Cy5-pCP was captured by lysosomes within 1 h (the red fluorescence from Cy5-CP overlays the green one from lysotracker blue), and a large amount of Cy5-CP escaped from the lysosomes (the red fluorescence from Cy5-pCP separated from the blue one from lysotracker blue) at 4 h, with 90% Cy5-pCP in the plasma (Fig. 3C and S16). The efficient lysosome escape is attributed to the “proton sponge effect” [37]. In the acidic lysosomes, the protonation of PEI in ZEBRA increased, which stimulated the influx of protons and chloride ions, increasing osmotic pressure within lysosomes [38]. The cationic ZEBRA together with osmotic vesicle swelling, led to the disruption of the lysosome membrane, followed by the release of plasmids into the cytosol. The effective cellular uptake and lysosomal escape imply that ZEBRA facilitates the cellular uptake of pCP and protects them from degradation by the enzymes in the lysosome.

3.4. Inhibition of tumor cells induced by Cas9-based polyplexes in vitro

We evaluated the transfection efficiency of the Cy5-ZEBRAP in other different cell lines including MDA-MB-231, MCF-7,
the treatment of the cells by ZEBRAP. The pretreatment of the anti-CD44 antibody on the HeLa cells significantly reduced intracellular accumulation of the ZEBRAP, and the cellular uptake was reduced significantly (CRP=59.7%; MFI=1833) (Fig. S17). This confirms the significant role of the HA residue in mediating the specific targeting of CD44-positive cells.

We evaluated the transfection of pCP induced effect in vitro. The transfection with ZEBRAP induced significant GFP-positive cells (Fig. S18). WB analysis showed that the cells treated by the ZEBRAP expressed a high-level of Cas9 proteins (Fig. 4C). As expected, the inhibition of PLK1 induced by ZEBRAP was as high as approximately 95%, which was much higher than those of other groups (Fig. 4C). The inhibition of PLK1 resulted in ~80% of the apoptosis by ZEBRAP, which was much higher compared with the control groups (Fig. 4D). T7E1 assay indicated that the ZEBRAP induced effective PLK1 gene editing, which was approximately 2-fold higher than the LCP-treated one (Fig. 4E and S19). But other groups, such as NC, ZEBRA, pCP, and ZEBRAL (ZEBRA/Cas9-sgLuciferase) showed no detectable cutting. We further characterized the off-target effects of the ZEBRAP. In 10 sites with high similarity to the target gene, no significant off-target effect was detected (Fig. 4F), which was consistent with the Sanger sequencing analysis (Fig. S20). These results imply that ZEBRA can specifically edit the target gene without detectable off-target effects, which induced a high level of cancer cell apoptosis and suppression.

3.5. Tumor inhibition evaluations

We evaluated the performance of ZEBRAP in vivo by intratumoral injection. After 16 d of treatment, the tumor volumes of the ZEBRAP group were approximately 1/20 of the saline-treated one (Fig. 5A&B), which was also confirmed by the tumor weight (Fig. 5C). In contrast, other groups including saline, ZEBRA, CP, ZEBRACL, and LCP group-treated tumors showed negligible inhibition effects on the tumor growth (Fig. 5A&5B). Lipo2000 has used a positive control, for Lipo2000 was a commercial transfection reagent and used commonly both in vitro and in vivo [5,14]. We analyzed the expression of PLK1 by WB and found that ZEBRAP induced effective inhibitory effects on PLK1 expression (Fig. 5D), resulting in the significant apoptosis of the tumor cells (Fig. 5E). We further analyzed the CD44 expression in different tissues and found that the tumor tissues showed a much higher level of CD44, compared with the muscle or skin tissues (Fig. S21). PA imaging analysis indicated that ZEBRA enabled the improvement of the accumulation of plasmids in the CD44-positive tumors (Fig. S22). The effective cancer cell apoptosis may be attributed to HA possessing a strong affinity for CD44 molecules, and thus improved the tumor-targeting significantly [39]. Haematoxylin and eosin (HE) staining of the extracted organs (the heart, liver, spleen, lung, and kidney) indicated that ZEBRAP showed no significant toxicity on the normal tissues (Fig. 5F). The evidence indicated that ZEBRA, as a vector, enabled to delivery of the pCP to induce efficient gene editing of tumor tissues.
3.6. Discussion

Chemotherapy is used as a common approach for cancer therapy at present [40]. However, chemotherapy usually leads to multiple side effects, such as drug resistance or high toxicity [41–43]. Gene therapy has emerged as an effective approach for the treatment of challenging diseases, including cancers. Especially, CRISPR/Cas9 showed great potential in this field. This powerful tool enabled cleaving the oncogenes precisely, which inhibited the expression of oncogenes and induced the apoptosis of cancer cells. There is in great demand to construct an efficient and safe vehicle for the delivery of the CRISPR/Cas9 system.

Several candidates can be considered for the purpose. Viral vectors showed excellent gene transfection efficiency. However, their application in gene delivery is still limited due to the problems it causes, including inflammation and immunogenicity [8]. Non-viral vectors, such as cationic polymers and lipids, are promising alternatives. The positive liposomes showed efficient transfection in multiple cell lines. However, previous studies showed its instability in serum and highly cytotoxic profile without appropriate modifications [44]. Polymeric vectors, are broadly considered as a promising choice for efficient and targeted gene delivery. Previous studies confirmed that high molecule weight (HMW) PEI 25 kDa showed high transfection efficiency, however, it at the same time exhibited significant cytotoxicity due to its high charge density and retention of non-degradable HMW PEI in cells [45]. The LMW PEI (molecular weight <2 kDa), in contrast, showed much lower cytotoxic but at cost of its transfection efficiency. To take advantage of the low toxicity of LMW PEI molecules, much effort has been devoted to tailoring the PEI molecules to form a series of derivatives that have a chemistry architecture that can condense large DNA molecules yet remains low cytotoxicity.

In this study, a novel zwitterionic polymer-inspired material, ZEBRA was endowed with multifunction and could be considered as a useful tool for CRISPR/Cas9 delivery. ZEBRA...
was composed of AG, LMW PEI, and hyaluronic acid, and the components function synergistically. ZEBRA induced a high level of cellular uptake, which was achieved by the incubation for a short time (Fig. 3A and 3B). Furthermore, the conjugation of LMW PEI increased the charge density and improved the lysosome escape of plasmids after the polypelexes were formulated (Fig. 3C). These properties ensured the high-level expression of Cas9 proteins, which was extremely important for successful gene editing (Fig. 4C). To reduce the non-specific delivery, vehicles with cell selectivity were preferred for nucleic acid delivery. The HA-receptor, CD44 is usually found to be overexpressed in the malignant cells [26], and it is reasonable to assume that HA-decorated vector will preferentially attach to these cells. The data indicated that HeLa cells and MDA-MB-231 cells expressed a high level of CD44 (Fig. 4A). We designed the HA block on the ZEBRA to realize preferential tumor accumulation and increase the cellular uptake, which was confirmed to be effectual. Different from the materials with a positive charge, ZEBRA showed reduced protein adsorptions, which was attributed to the hydrophilicity and steric hindrance of HA, effectively resisting the nonspecific adsorption from biomolecules HA. The gelling property of the AG block was designed to promote gene compaction. Also, the abundant hydroxyl in the AG block may provide general attachment sites for conjugating PEI. Based on the characteristic of polysaccharide-derived ZEBAR, the CRISPR/Cas9 system was delivered to the CD44-positive cancer cells efficiently both in vitro and in vivo (Fig. S12, S14, and S22), and induced significant gene editing and apoptosis (Fig. 4D and 4E), conferring to the inhibition of tumor cell growth. Interestingly, the gene-editing of Plk1 did not cause visible off-target, implying the precise gene editing of CRISPR/Cas9. The polysaccharide-derived ZEBRA showed great potential in the field of gene editing.

4. Conclusions

In conclusion, a novel zwitterionic polymer-inspired material, ZEBRA was synthesized for specific delivery of CRISPR/Cas9 system. ZEBRA showed low cytotoxicity, biodegradability, and ability to mediate efficient gene editing. Especially, ZEBRA showed much-improved transfection efficiency in CD44 positive cells. More importantly, ZEBRA enabled to delivery large size plasmids encoded with Cas9 and sgRNA targeting to Plk1 gene, inducing remarkable cancer cell apoptosis and tumor cell inhibition both in vitro and in vivo. The zwitterionic polymer-inspired material, ZEBRA opened a new avenue in the field of cancer therapy by inducing efficient gene editing.

Conflicts of interest

The authors have no conflicts of interest to declare.
Acknowledgments

We thank National Natural Science Foundation of China (82072047, 81700382), Natural Science Foundation of Guangdong Province (2019A151012166), Research Foundation of Education Bureau of Guangdong Province (2021ZDZX2004), Basic and Applied Basic Research Project of Guangzhou (02080390), Outstanding Youth Development Program of Guangzhou Medical University.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ajps.2022.08.001.

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