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"Building-block crosslinking" micelles for enhancing cellular transfection of biocompatible polycations

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ABSTRACT **Incorporating functional ligands and biodegradable bonds into biocompatible low-molecular-weight (LMW) polymers, such as 1.8 kDa poly(ethylenimine) (PEI 1.8k), is a common strategy to improve the properties of LMW polymers including biosafety and delivery efficacy. This study demonstrates the hypothesis that introducing different functional ligands and linked reductive disulfides in PEI 1.8k will achieve superior siRNA transfection efficiency. By incorporating PEI-X (X represents cholesterol (Ch), heptafluorobutyric anhydride (HFBA, F) and 4-carboxyphenylboronic acid (PBA)) functional ligands into PEI 1.8k and subsequently crosslinking with each other *via* disulfide bond links, reductive-responsive PEI-X-SS-X-PEI copolymers were constructed to enhance the cellular transfection *via* the synergistic effect of the high affinity of Ch, F and PBA to cell membranes and the disulfide reduction triggered intracellular disassembly of micelles and subsequent siRNA release. Extraordinarily, ternary Ch-SS-F-SS-PBA micelles exhibited the strongest siRNA transfection efficiencies in *in vitro* cell experiments and *in vivo* animal experiments due to the coordination of enhanced serum stability, promoted cell uptake and endosomal escape, and cell targeting ability. This strategy of constructed multifunctional polymer here we called "building-block crosslinking" showed a simple and smart way to synthesize new materials. Also this strategy of constructing ligands-directed reduction-sensitive micelles improves the transfection efficiency of LMW PEI and provides a valuable insight to develop novel gene delivery systems.**

Keywords: siRNA delivery, ligand modification, low-molecular-weight polymers, transfection efficiency

INTRODUCTION

RNA interference (RNAi) therapy has attracted significant attention over the past two decades due to its superior therapeutic efficacy for various diseases, from gene-related disorders to cancer. However, the electronegativity of siRNA is a main barrier for it to enter the cells, which has limited their application in clinic [1–3]. Therefore, various strategies have been tried to overcome the barriers by developing cationic polymer-based siRNA delivery systems, such as poly(ethylenimine) (PEI) or polycatechols inspired by natural polyphenols [4–7]. These materials can form polyelectrolyte complexes with siRNA, termed polyplexes, then protect siRNA from degradation and facilitate their transport across cellular membranes [8,9].

The dilemma between the high toxicity caused by highly positive charge density and low transfection efficacy is an obstacle for the application of cationic polymers in siRNA delivery [2,10,11]. Many functional groups have been incorporated into the siRNA delivery system for improving the transfection efficiency such as lipids, fluorides, sugar, peptides, or antibodies [12–14]. To date, a variety of ligands containing different characteristics have been developed; however, more are needed to be discovered urgently. Among them, co-modification of polymers with two functional ligands is a potential strategy to improve the siRNA transfection efficiency, and meanwhile, decrease the toxicity, such as polymers modified by polyethylene glycol (PEG) together with hydrophobic ligand [15], PEG with fluorination [16], or phenylboronic acid (PBA) with cholesterol (Ch) [17]. However, nowadays the synthesis of ligand-modified

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polymers is mainly based on the conjugation of one by one ligand to one polymer material, resulting in complex synthesis steps.

Introduction of biodegradable bonds such as disulfide (–S–S–) linkage is a classic strategy to overcome the toxicity and enhance the transfection efficacy of cationic polymers as the existing redox potential gradient between the extracellular environment and subcellular organelles [8,18,19]. The reductive environment-sensitive –S–S– crosslinked polymers were stable outside the cell and destabilized in the intracellular environment to facilitate siRNA release.

Here, we propose a "building-block crosslinking" strategy, i.e., co-introduction of ligands and bioreducibility features in a single polycation, which will dramatically improve the transfection efficiency while reducing their toxicity. Firstly, we screened a library of functional ligands with different properties. In a comprehensive analysis, Ch, heptafluorobutyric anhydride (HFBA, F) and 4-carboxyphenylboronic acid (PBA) were selected and expected to satisfy our expectation because of their different hydrophilic to hydrophobic segment ratio and different contributions to improve siRNA delivery. Ch has been frequently used as a good lipid anchor to improve the cellular uptake and stability of the polymer possibly *via* its biocompatibility with membrane phospholipids [17,20]. Fluorine is believed to be able to facilitate cell uptake as well as endosomal escape of the polymer due to its hydrophobic and lipophobic characteristic, therefore showing a high tendency towards phase separation in both polar and nonpolar environments [21–23]. Boronic acid incorporated into the polymer is reported to possess multiple properties, such as cell targeting, cellular uptake, endosomal escape and cytosolic cargo release [24–28]. Then, PEI-X (X represents Ch, F and PBA) monomer was synthesized by introducing Ch, F and PBA in PEI with a molecular weight (M_w) of 1.8 kDa (PEI 1.8k), respectively. Subsequently, bioreducible PEI-X-X-PEI copolymers with cystamine bisacrylamide (CBA) as the cross-linker were created by a typical Michael polymerization, including one-ligand modification (Ch-PEI-SS-Ch-PEI (Ch-SS-Ch), F-PEI-SS-F-PEI (F-SS-F) and PBA-PEI-SS-PBA-PEI (PBA-SS-PBA)); two-ligand modification (Ch-PEI-SS-F-PEI (Ch-SS-F), Ch-PEI-SS-PBA-PEI (Ch-SS-PBA) and F-PEI-SS-PBA-PEI (F-SS-PBA)); and three-ligand modification (Ch-PEI-SS-F-PEI-SS-PBA-PEI (Ch-SS-F-SS-PBA)) (Fig. 1 and Fig. S1).

In this study, transfection efficiencies of all polymers were evaluated and compared across several cell lines *in vitro*, including CHO, B16F10, 4T1 and MDA-MB-231cells, as well as an *in vivo* B16F10 xenograft model. Robust/efficient gene transfection and serum resistance of the Ch-SS-F-SS-PBA copolymer have been eventually verified in various cell lines. Therefore, our strategy offers a novel and versatile perspective in the design of high-performance gene transfection materials.

EXPERIMENTAL SECTION

Materials

Branched PEI (M_w 1.8 and 25 kDa), cholesteryl chloroformate, *N*,*N*-diisopropylethylamine (DIPEA), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and PBA were purchased from Aladdin (China). CBA and HFBA were purchased from Sigma-Aldrich (USA). All siRNA were purchased from GenePharma (China). All other reagents were from Nanjing Wanqing Chemical Glassware Instrument unless otherwise stated.

Synthesis and characterization of PEI-X monomers and crosslinked PEI-X-SS-X-PEI copolymers

PEI-Ch, PEI-F and PEI-PBA monomers with different degrees of substitution were synthesized according to previously reported procedures, respectively [20,29,30]. For the synthesis of PEI-Ch, PEI (100 mg) was first dissolved in a mixture of anhydrous methanol and DIEPA. Different calculated amounts of cholesteryl chloroformate (25 and 50 mg) in anhydrous methylene chloride were added to the ice-cold PEI solution drop by drop over 1 h. The reaction was continued under stirring for another 24 h. The product was obtained by evaporating the solvent and washing with diethyl ether three times to remove the unreacted cholesteryl chloroformate.

To fluorinate PEI, PEI (100 mg) was first dissolved in methanol and mixed with different amounts of HFBA (45 and 90 mg) and trimethylamine (15 and 30 mg), respectively. The reaction mixture was stirred at room temperature for 48 h.

PEI-PBA was synthesized by a typical reaction of amide bonds using carbodiimide chemistry. Briefly, different amounts of PBA (9 and 36 mg) were dissolved in 5 mL mixture solvent (CH_3OH/H_2O=1:1, *v*/*v*) and then corresponding amounts of EDC and NHS (4.0 molar equivalent of PBA) were added into the mixtures, which reacted at room temperature for 1 h in order to activate the carboxyl group. The resulting solution was dropped into PEI (100 mg) solution and kept stirring at room temperature for 24 h.

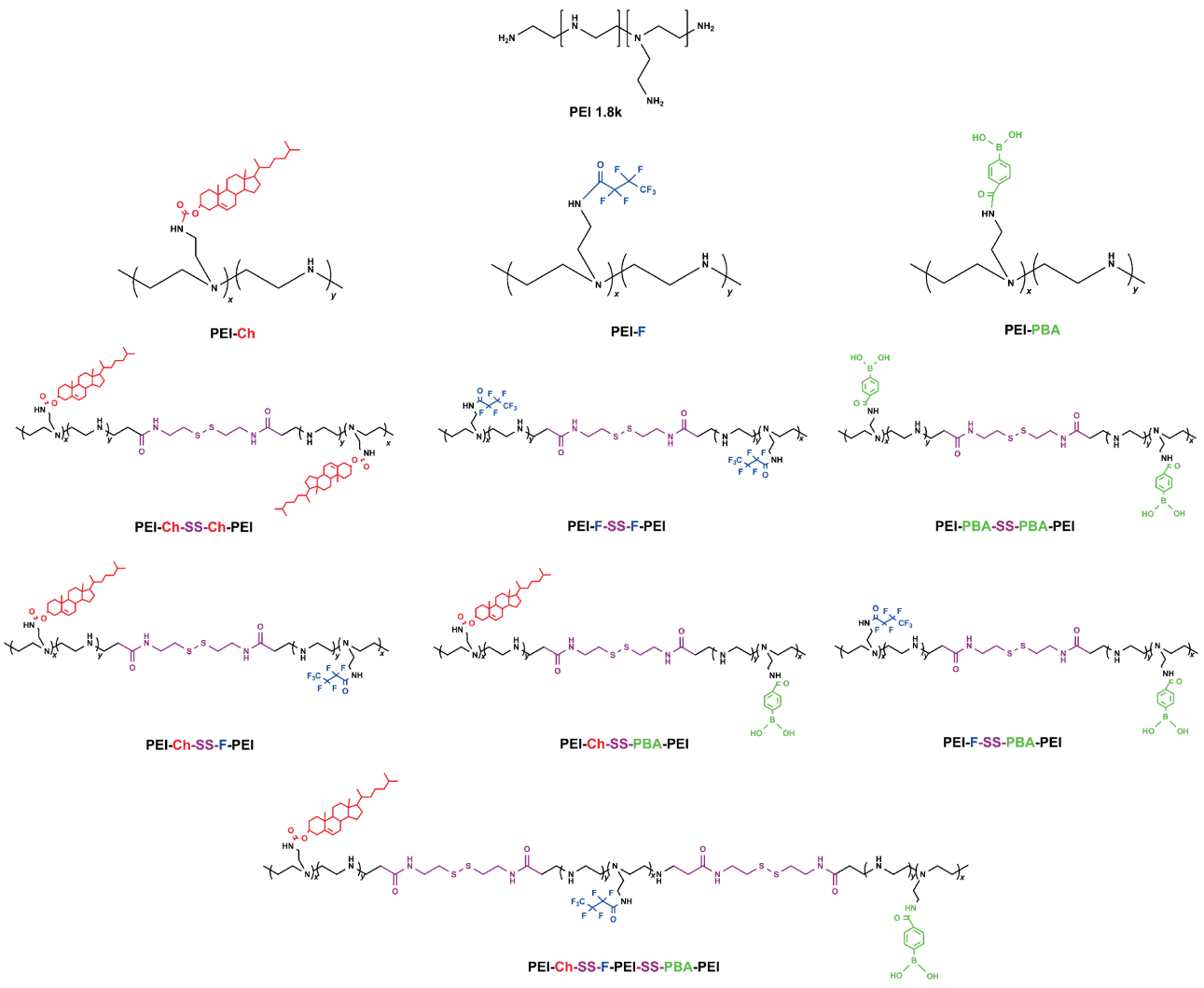

Figure 1 Structures of PEI-X monomers and crosslinked PEI-X-SS-X-PEI copolymers.

All above products were purified by extensively dialyzing against deionized water (molecular weight cut off 1000 Da). The cholesteryl content and PBA content were determined by ^{1}H nuclear magnetic resonance (^{1}H NMR; Bruker 500 MHz) and analyzed by TopSpin 3.5pl6 software. The fluorine content was determined by fluorine elemental analysis and confirmed by ^{1}F NMR.

PEI-X-SS-X-PEI copolymers were synthesized by Michael-type polyaddition of CBA and PEI-X monomers as described by previous studies. Briefly, for one-ligand modification (Ch-SS-Ch, F-SS-F and PBA-SS-PBA), PEI-X (0.1 molar equivalent of PEI) and 0.1 mol CBA were dissolved in methanol/water mixture (4 mL, 7:3 *v*/*v*); for two-ligand modification (Ch-SS-F, Ch-SS-PBA and F-SS-PBA), equivalent mole ratio of PEI-X (like 0.05 mol L^{-1} PEI-Ch and 0.05 mol L^{-1} PEI-F) and 0.1 mol CBA were dissolved in methanol/water mixture (4 mL, 7:3, *v*/*v*); for three-ligand modification (Ch-SS-F-SS-PBA), PEI-Ch (0.033 mol L^{-1}), PEI-F (0.033 mol L^{-1}), PEI-PBA (0.033 mol L^{-1}) and 0.1 mol CBA were dissolved in methanol/water mixture (4 mL, 7:3, *v*/*v*). All polymerization was carried out for 72 h at 37°C. It should be noted that, for two or three-ligand modification, PEI-X1 was first added to the reaction system, and then PEI-X2 was added after 6 h, and then PEI-X3 (if has) was added after another 6 h to control the polymer synthesis. Then, the products were isolated by freeze-dryer after dialysis. The molecular weights of these copolymers were tested by gel permeation chromatography (GPC) with 0.1 mol L^{-1} sodium acetate buffer (pH 5.0) as the running eluent, and the number-average molecular weights (M_n) of Ch-SS-Ch, F-SS-F, PBA-SS-PBA, Ch-SS-F, Ch-SS-PBA, F-SS-

PBA and Ch-SS-F-SS-PBA were 23.4, 26.8, 22.1, 25.4, 22.6, 24.8 and 24.3 kDa, respectively.

Preparation and characterization of polyplexes

The polyplexes were prepared by mixing equal volume of siRNA solution (20 μg mL^{-1} in 10 mmol L^{-1} 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer, pH 7.3) with each polymer and incubating the mixture at room temperature for 30 min to achieve the desired *w*/*w* ratios. The composition of the siRNA polyplexes in this study was expressed as equivalent PEI/siRNA ratios, excluding the Ch, HFBA and PBA contents. The siRNA binding ability of each polymer to condense siRNA was evaluated by agarose gel electrophoresis ran for 15 min at 100 V in 0.5× tris/borate/ethylene diamine tetraacetic acid (EDTA) buffer. The size and zeta potential were measured by Zeta Plus (Brookhaven, USA). The morphology was observed by transmission electron microscopy (TEM, H-600, Hitachi, Japan). The colloidal stability of Ch-SS-F-SS-PBA/siRNA polyplexes was investigated in phosphate-buffered saline (PBS, pH 7.4) with or without 10% fetal calf serum (FBS) at room temperature.

Cell culture

Chinese hamster ovary (CHO) cell, murine melanoma cell line B16F10 and mouse breast cancer cell line 4T1 were maintained in RPMI-1640 medium with 10% FBS. The human breast cancer cell line MDA-MB-231 were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS.

MTT assay

The cytotoxicity of the polymers and polyplexes was measured by a well-established (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in B16F10 cells. Briefly, the cells were seeded in 96-well plates at a density around 10^4 cells per well overnight. The cells were incubated with the polymers for 24 h and the polyplexes for 4 h. After that, the incubation media were removed and replaced with fresh media, and then a standard MTT assay was used to determine the cytotoxicity.

Cell uptake and intracellular trafficking

The cells were seeded in 24-well plates and cultured to 60% confluence. Then the polyplexes were prepared with FAM™ dye-Labeled miRNA (FAM-miRNA) (100 nmol L^{-1}) for 4 h. The cells were then trypsinized and the uptake was analyzed by BD FACSCalibur flow cytometer. Intracellular distribution was assessed by confocal microscopy following DAPI staining 4 h after incubation. In some experiments, the lysosomes were stained with LysoTracker™ Red before imaging.

Reporter gene silencing *in vitro*

To evaluate and compare the gene transfection of PEI-X monomers and crosslinked PEI-X-SS-X-PEI copolymers, cells (CHO-EGFP, B16F10.Luc, 4T1.Luc and MDA-MB-231-Luc) were seeded in the plates at a density of 10,000–40,000 per well and cultured until 70%–80% confluence. Polyplexes prepared with siEGFP or siLuc at different *w*/*w* ratios were added into the wells (100 nmol L^{-1} siRNA) with or without FBS and incubated for 4 h. The polyplexes were replaced with fresh medium containing 10% FBS for another 24 h incubation. After that, the expression of EGFP cells was measured and observed by flow cytometry and confocal microscopy. The Luc cells were washed with PBS and lysed with cell lysis buffer. The Luc activity was measured with a Luc assay kit according to the manufacturer's instructions. The relative light units (RLU) were measured and normalized to the cell lysate protein concentrations measured by a BCA protein assay kit.

Luc gene silencing *in vivo*

Six-week-old female C57BL/6 mice were used in compliance with approved protocol by the Institutional Animal Care and Use Committee of China Pharmaceutical University. B16F10 cells (1×10^5) stably expressing luciferase were subcutaneously injected into the mice. The *D*-luciferin potassium salt (200 μL, 15 mg mL^{-1}) was administered intraperitoneally to anesthetize the mice until the tumor size reached 100 mm^3. After the Luc bioluminescence was measured and recorded on day 0 by using a Tanon 5200 Multiimaging system (Tanon Science & Technology Inc., China), the mice were then divided into seven groups (three mice per group) and administered with the polyplexes by intratumoral injection (100 μL, 1.2 mg kg^{-1} siRNA, *w*/*w* 2 for PEI 25k, *w*/*w* 3 for other polymers). The treatments were repeated on day 1 and bioluminescence of the tumors was measured on day 2. In the end, the mice were euthanized, and the tumors were excised and homogenized in a lysis buffer followed by centrifugation at 12,000 ×*g* for 10 min for the detection of Luc expression.

Statistical analysis

Statistical assessment was conducted by two-sided Student's *t* test for two groups and one-way ANOVA analysis of variance for multiple groups ($P<0.05$ was considered statistically significant). All analyses were performed with

SPSS 19.0 for Windows.

RESULTS AND DISCUSSION

Characterization of functionalized PEI-X monomers and PEI-X-X-PEI copolymers

PEI has been widely used for gene delivery because numerous amino groups on its scaffold endow it with the ability to condense nucleic acids and facilitate their endosomal escape [31,32]. However, its delivery efficiency mainly depends on the N/P ratio, which means that higher transfection efficiency with higher net-positive surface charge as well as an excess of free cationic polymers within the mixture results in severe toxicity [33]. Hence, incorporation of functional ligands and responsive linkages into low-molecular-weight (LMW) PEI represents feasible strategies to balance its toxicity and transfection efficiency [34].

In this study, we first synthesized three functionalized PEI monomers by modifying PEI 1.8k with Ch, F and PBA, respectively. The products are termed as PEI-X1 and PEI-X2 with increasing content of the functional ligands. Their siRNA delivery ability was subsequently determined and the results showed that the polyplexes prepared by PEI-X2 with high content were more effective in uptake and gene silencing activity than PEI-X1 with low content for the same ligand modification and parent PEI 1.8k (i.e., PEI-Ch2>PEI-Ch1>PEI 1.8k, PEI-F2>PEI-F1>PEI 1.8k, PEI-PBA2>PEI-PBA1>PEI 1.8k) (Fig. 2a, b). Second, bioreducible PEI-X-X-PEI copolymers were synthesized by crosslinking of PEI-X2 with CBA (PEI-X-SS-X-PEI), including one-ligand modification (Ch-SS-Ch, F-SS-F and PBA-SS-PBA), two-ligand modification (Ch-SS-F, Ch-SS-PBA and F-SS-PBA) and three-ligand modification (Ch-SS-F-SS-PBA).

The contents of Ch and PBA in the monomers were determined from ^{1}H NMR integral intensity of the Ch methyl group at 0.65 ppm and the phenyl proton signal at 7.5–8.0 ppm, respectively. The fluorine content was determined by elemental analysis and confirmed by ^{1}F NMR. The detailed characterization information of these polymers is shown in Table S1, and Figs S2–S6.

Based on the different hydrophobicity of Ch, F and PBA, we then prepared the solutions of all monomers and copolymers in HEPES (10 mmol L^{-1}, pH 7.4) at a final concentration of 1 mg mL^{-1} and measured their particle sizes by dynamic light scattering (DLS). As shown in Fig. S7, PEI 1.8k, PEI-F2, PEI-PBA2, F-SS-F, PBA-SS-PBA and F-SS-PBA did not exhibit measurable particle sizes, but PEI-Ch2, Ch-SS-Ch, Ch-SS-F, Ch-SS-PBA and Ch-SS-F-SS-PBA showed the diameters of 385.4, 295.9, 233.4, 199.6 and 154.3 nm, respectively. The siRNA binding ability of the above monomers and crosslinked copolymers was firstly evaluated, as shown in Fig. S8. These crosslinked copolymers generally exhibited enhanced siRNA packaging ability comparable to the corresponding PEI-X monomers, which indicated by fully condensed siRNA at or less *w*/*w* ratio of 1.0.

Hydrodynamic sizes and zeta-potentials of Ch-SS-F-SS-PBA/siRNA polyplexes prepared at various *w*/*w* ratios were measured and the results are shown in Fig. S9. The size of the prepared polyplexes increased gradually with increasing *w*/*w* ratio from 160 nm at *w*/*w*=1 to 246 nm at *w*/*w*=9. As expected, increasing the *w*/*w* ratio also resulted in an increase of the zeta potential, which ranged from 21 mV at *w*/*w*=1 to 42 mV at *w*/*w*=9. The formed Ch-SS-F-SS-PBA/siRNA polyplexes prepared at *w*/*w*=3 were spherical. We also tested the colloidal stability of the Ch-SS-F-SS-PBA polyplexes by incubation in PBS (pH 7.4) with or without 10% FBS at room temperature and we found the polyplexes could maintain their initial size for at least 24 h (Fig. S10).

Ch-SS-F-SS-PBA copolymer improves transfection efficacy of PEI 1.8k

As expected, the cell viability of the PEI-X-SS-X-PEI copolymer was slightly changed compared with the corresponding PEI-X monomers, which was reflected in their similar value of half maximal inhibitory concentration (IC_{50}) since disulfide bonds exist in the copolymers but not in the monomers (Table S2). To determine the safety *w*/*w* ratios for the use of gene transfection, the cytotoxicity of polyplexes was evaluated by mixing siScr and polymers. As shown in Fig. 2c, PEI-Ch/siRNA polyplexes and Ch-SS-Ch/siRNA polyplexes showed the highest toxicity with the cell viability remaining about 66% and 61% up to *w*/*w* 9, respectively. All PEI-X monomers and crosslinked PEI-X-SS-X-PEI copolymer polyplexes could almost maintain their cell viability with 80% at *w*/*w* 6; by contrast, PEI 25k polyplexes caused nearly 54% cell death at *w*/*w*=3.

As mentioned above, the use of *w*/*w* ratios was restricted to *w*/*w* 2 for PEI 25k polyplexes and *w*/*w* 3 for other polyplexes in the following biological studies. Here, our co-modified copolymer of Ch-SS-F-SS-PBA with three ligands significantly improves the EGFP transfection efficiency compared with the copolymers (Ch-SS-F, Ch-SS-PBA, F-SS-PBA) with two-ligand modification, copolymers (Ch-SS-Ch, F-SS-F, PBA-SS-PBA) with single-ligand modification, PEI monomers and PEI 25k in CHO-EGFP cells. Surprisingly, co-modified copoly-

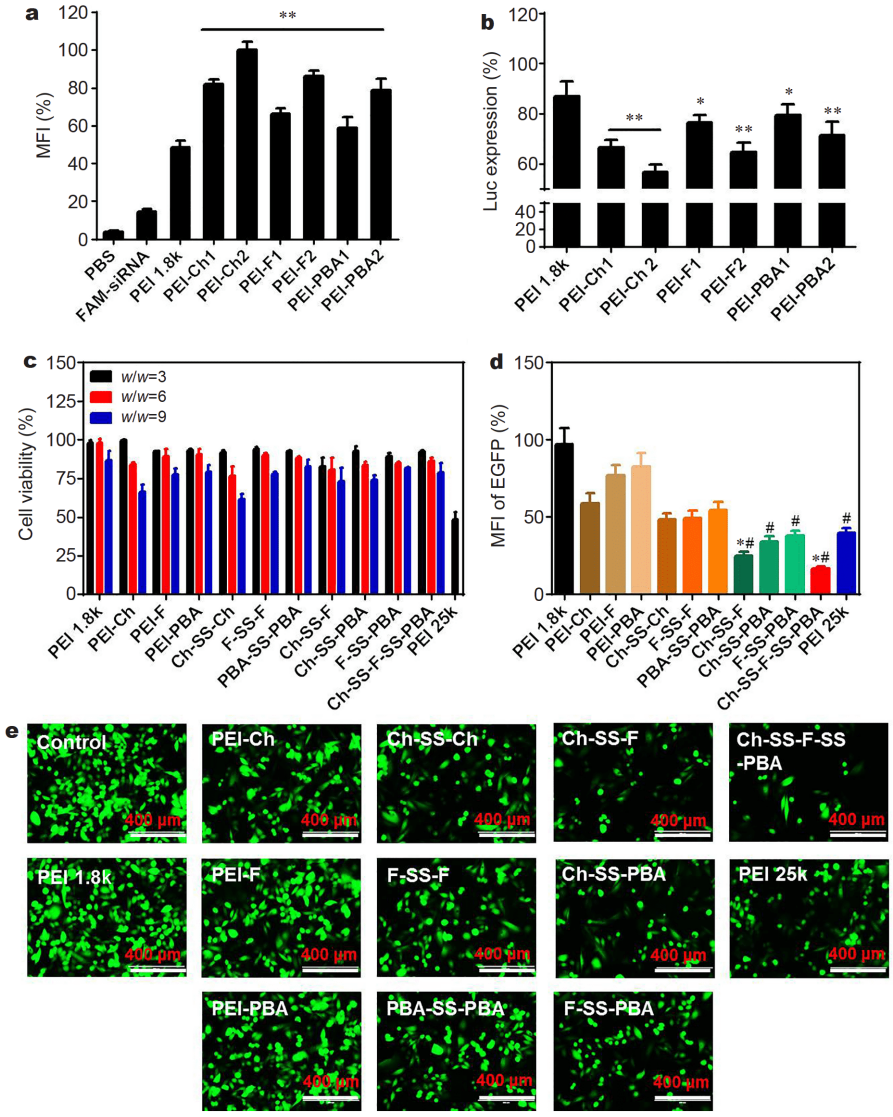

Figure 2 Transfection efficiency of the polyplexes. The cell uptake (a) and gene silencing ability (b) of PEI monomers. $^{*}P<0.05$ and $^{**}P<0.01$ *vs.* PEI 1.8k group. (c) Cell viability of all the tested siRNA polyplexes in CHO cells. EGFP gene silencing efficacy was determined by flow cytometry (d) and confocal microscopy (e) in CHO-EGFP cells. Scale bar=400 μm. $^{*}P<0.05$ *vs.* PEI 25k group; $^{\#}P<0.05$ *vs.* Ch-SS-Ch group. The results are shown as mean±standard deviation (SD) (n=3).

mers with two ligands, especially, Ch-SS-F also exhibited satisfying EGFP silencing ability compared with other polymers (Fig. 2d, e).

To further validate the results with EGFP silencing ability in other target gene and cell lines, we tested the transfection efficiency of the Ch-SS-F-SS-PBA copolymer in B16F10, 4T1 and MDA-MB-231 cells with Luc expression. As shown in Fig. 3, the Ch-SS-F-SS-PBA copolymer achieved the best transfection activity when compared with other crosslinked copolymers and PEI 25k polyplexes even at a lower *w*/*w* ratio in B16F10 cells. In addition, we evaluated the efficiency of Ch-SS-F-SS-PBA copolymer in 4T1 and MDA-MB-231 cells, which are typically difficult to transfect [35]. The results showed that the Ch-SS-F-SS-PBA copolymer achieved Luc silencing at ~40% in both two cells at *w*/*w* 3, while PEI 25k exhibited weak Luc silencing of ~80% at *w*/*w* 2. These results suggest that Ch-SS-F-SS-PBA is an efficient and biocompatible vehicle for introducing both EGFP and luciferase genes into a list of commonly used cells, including those significant but hard-to-transfect cell types.

Transfection mechanisms of Ch-SS-F-SS-PBA copolymer

To verify the potential factors behind the robust trans-

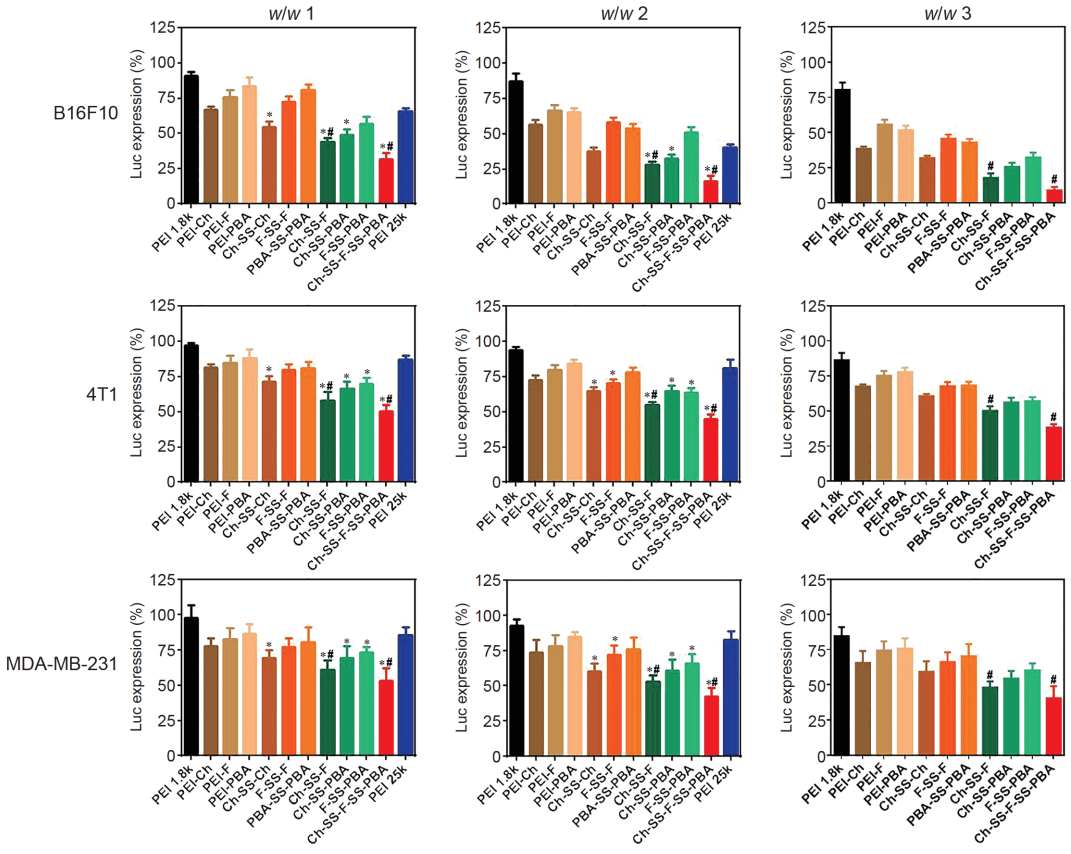

Figure 3 Luciferase reporter gene silencing. Transfection efficacy of siLuc polyplexes in B16F10.Luc, 4T1.Luc and 231.Luc cells at different *w*/*w* ratios. * $P<0.05$ *vs*. PEI 25k group; # $P<0.05$ *vs*. Ch-SS-Ch group. The results are shown as mean±SD ($n=3$).

fection efficiency of the Ch-SS-F-SS-PBA copolymer, cellular uptake and intracellular trafficking behaviors of PEI 25k/siRNA (*w*/*w* 2), Ch-SS-Ch/siRNA (*w*/*w* 3), Ch-SS-F/siRNA (*w*/*w* 3) and Ch-SS-F-SS-PBA/siRNA (*w*/*w* 3) polyplexes were investigated based on their transfection efficiency ability. As shown in Fig. 4a, the Ch-SS-F-SS-PBA copolymer exhibited the highest cellular uptake as reflected in the strong FAM fluorescence, and showed more efficient escape from the acidic vesicles stained by LysoTracker Red. In contrast, PEI 25k polyplexes showed weak FAM-siRNA fluorescence and less siRNA release. The efficient cellular uptake of Ch-SS-F-SS-PBA polyplexes was further confirmed by flow cytometry (Fig. 4b). The results suggest that introducing ligands with different properties on uptake is an effective strategy to enhance siRNA delivery.

Ch-SS-F-SS-PBA copolymer shows superior serum resistance

Serum resistance and stability of polyplexes were important features for gene delivery application. One of the main benefits of Ch and fluorination is to enhance the serum stability of polyplexes due to their unique natures, as their inactive surface against serum proteins [20,36]. Hence, we evaluated the effect of increasing serum content in the incubation media on the siLuc transfection activity of crosslinked copolymers based on the results of gene silencing in CHO, B16F10, 4T1 and MDA-MB-231 cell lines. As shown in Fig. 5, PEI 25k polyplexes displayed greatly decreased silencing activity with increasing serum content, while other polyplexes showed a certain degree of serum resistance and the resistance ability was as following: Ch-SS-F-SS-PBA>Ch-SS-F>Ch-SS-Ch>Ch-SS-PBA>F-SS-PBA. These results demonstrated that co-introduction of functional chains could improve the siRNA delivery efficiency *in vivo* when compared with the parent polymers or single modification.

Luc gene silencing *in vivo*

To further investigate whether the Ch-SS-F-SS-PBA copolymer can achieve better silencing effect *in vivo*, we have established a subcutaneous B16F10.Luc tumor

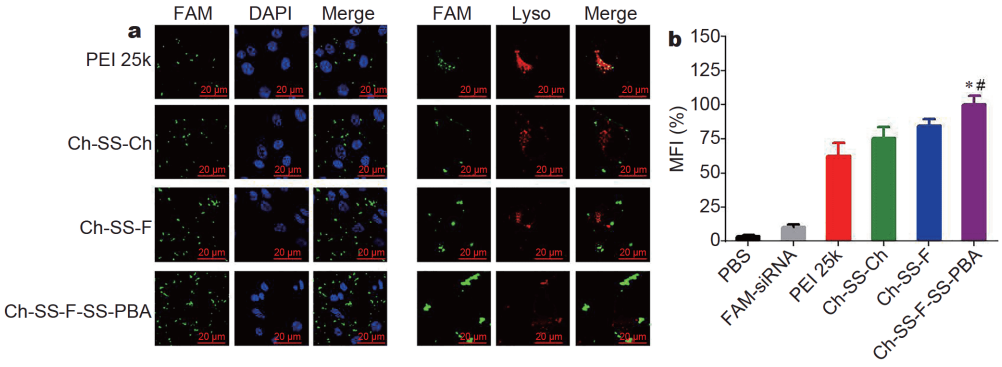

Figure 4 Cellular uptake and intracellular trafficking of Ch-SS-F-SS-PBA/siRNA polyplexes in B16F10 cells. (a) Intracellular distribution and trafficking of Ch-SS-F-SS-PBA/siRNA polyplexes by confocal microscopy after 4 h of incubation. Scale bar=20 μm. (b) Cellular uptake of Ch-SS-F-SS-PBA/siRNA polyplexes by flow cytometry after 4 h of incubation. * $P<0.05$ *vs.* PEI 25k group; $^{\#}$ $P<0.05$ *vs.* Ch-SS-Ch group.

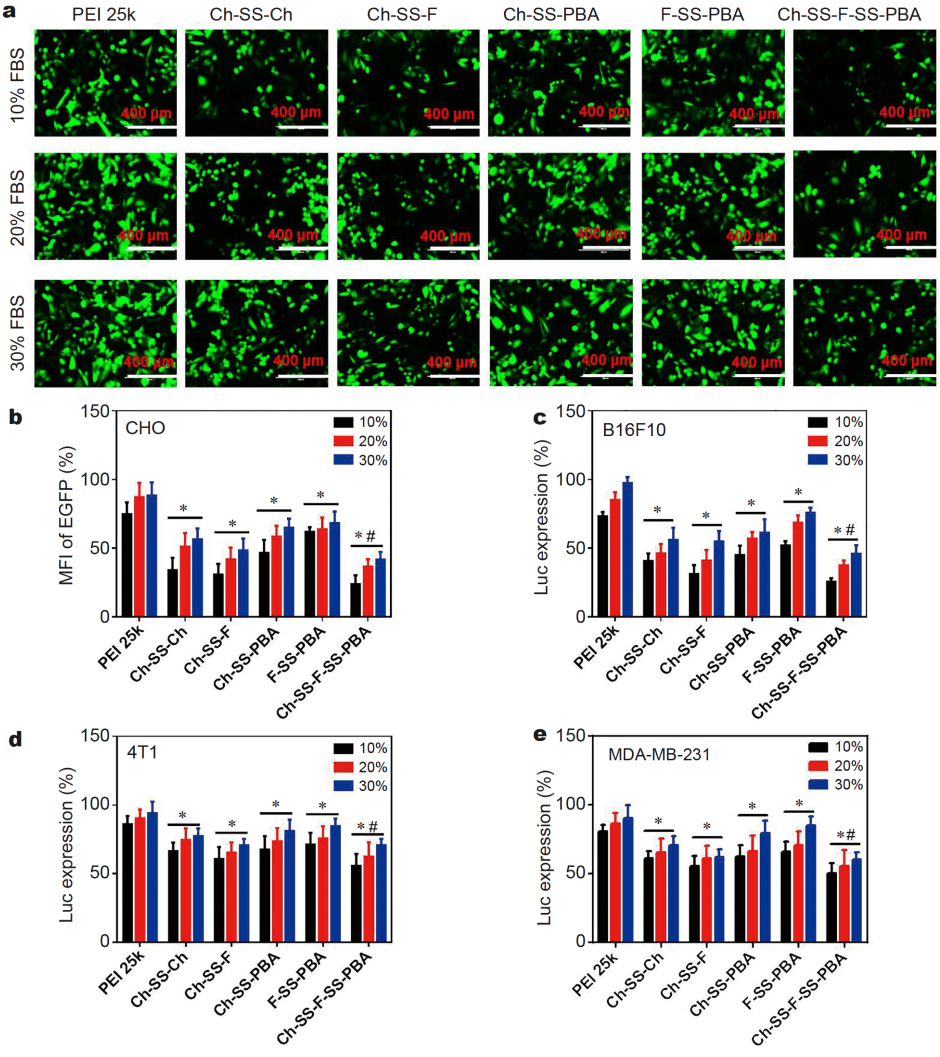

Figure 5 (a) Fluorescence images of EGFP protein expression on CHO-EGFP cells after different treatments in the presence of 10%, 20%, and 30% FBS detected by fluorescence microscopy. Scale bar=400 μm. Effect of different treatments on the transfection activity in CHO-EGFP (b), B16F10.Luc (c), 4T1.Luc (d) and MDA-MB-231.Luc (e) cells in the presence of 10%, 20%, and 30% FBS. * $P<0.05$ *vs.* PEI 25k group; $^{\#}$ $P<0.05$ *vs.* Ch-SS-Ch group. All results are shown as mean±SD (n=3).

model. The tumor bioluminescent images were taken and analyzed before and after treatment with the siLuc polyplexes by intratumoral injection. As shown in Fig. 6, control animals treated with PBS, free siLuc and siScr and limited Luc could silence about 0–8% (or no Luc silencing). Treatment with PEI 25k/siLuc, Ch-SS-Ch/siLuc, Ch-SS-F/siLuc and Ch-SS-F-SS-PBA/siLuc polyplexes resulted in the decrease of Luc expression in tumor after 2 d injection (Fig. 6a, b). In addition, the Ch-SS-F-SS-PBA/siLuc polyplexes exhibited the weakest fluorescence intensity compared with other polyplexes. To validate the *in vivo* bioluminescence result, the tumors were excised, homogenized and measured for Luc activity as above. As a result, Ch-SS-F-SS-PBA polyplexes showed 62% Luc silencing, while the Ch-SS-Ch and Ch-SS-F polyplexes showed 32% and 49% Luc silencing, respectively (Fig. 6c). In general, these results are consistent with the *in vitro* measurements, indicating that incorporating multiple ligands with different properties into the cationic polymer has great potential as siRNA delivery vehicle *in vivo*.

CONCLUSIONS

Introducing the functional ligands and cross-linking to LMW polymers with biodegradable linkage is the common strategy to improve their transfection efficiency. We designed a series of experiments to prove the superiority of co-modification "building-block crosslinking" strategy in polymers for gene transfection, which was reflected in the transfection efficiencies of Ch-SS-F-SS-PBA (three-ligand modified)>Ch-SS-F (two-ligand modified)>Ch-SS-Ch (single-ligand modified)>PEI-X monomers. These findings reveal the huge advantages of the strategy of combining functional ligands and bioreducibility in improving transfection efficiency and decreasing toxicity of polymers. This strategy provides a new vision for the design of LMW polymers by modification with functional ligands and bioreducibility.

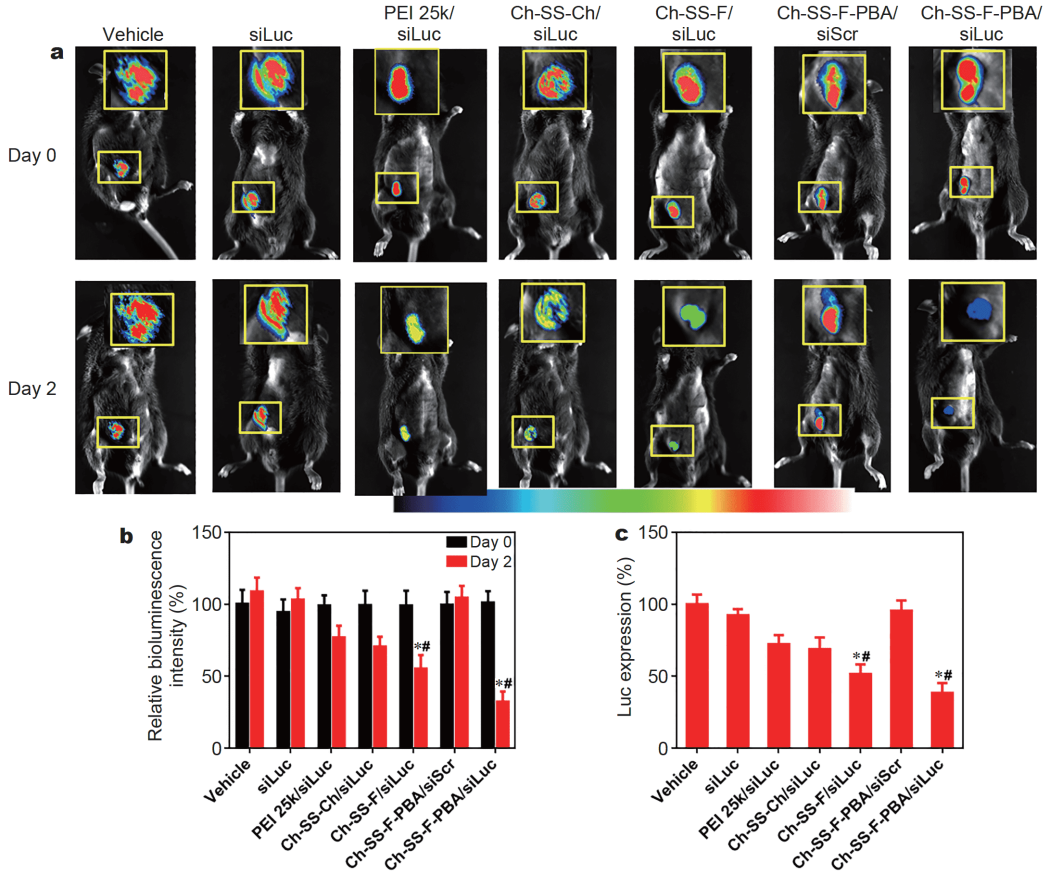

Figure 6 (a) Bioluminescence images of mice with B16F10.Luc tumors before and after gene silencing. (b) Quantification of Luc expression from the whole-body images. (c) *Ex vivo* analysis of the Luc activity in the isolated tumor tissues. All Luc silencing data are shown as mean% Luc expression relative to vehicle±SD (n=3). $^{*}P<0.05$ *vs.* PEI 25k group; $^{\#}P<0.05$ *vs.* Ch-SS-Ch group.

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Author contributions Wu P and Wang K were responsible for the study design; Wang K and Ding D conceived the project and acted as project leader; Wu P and Yin S performed the actuation experiments; Wu P and Yin S were responsible for the manuscript writing; Wu P and Liu T were responsible for the data analyses. All the authors commented and revised the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Supplymentary information Supporting data are available in the online version of the paper.

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"嵌段交联"胶束策略用于增强生物相容性阳离子聚合物的胞内转染效率

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摘要 将功能性配体和生物可降解性键结合到生物相容性的低分子量(LMW)聚合物中, 如1.8k聚乙烯亚胺(PEI), 是提高LMW聚合物生物安全性和递送效率的常见策略. 本研究证明了在PEI 1.8k中引入不同功能性配体并使用还原性二硫键将其交联可获得超高siRNA递送效率. 通过将X (X代表胆固醇(Ch)、七氟丁酸酐(HFBA, F)和4-羧苯基硼酸(PBA))功能配体分别引入PEI 1.8k中得到PEI-X单体聚合物, 然后将三者通过二硫键相互交联可成功构建还原响应性PEI-X-SS-X-PEI共聚物. 利用Ch、F、PBA对细胞膜的高亲和力、还原性二硫键触发的细胞内胶束解体及随后siRNA释放的协同作用, PEI-X-SS-X-PEI可显著增强siRNA的细胞转染. 这种三元型Ch-SS-F-SS-PBA/siRNA形成的胶束在体外细胞实验和体内动物实验中均表现出最强的siRNA转染效率, 这得益于胶束具有增强的血清稳定性、提升的细胞摄取和溶酶体逃逸能力以及细胞靶向性的协同作用. 这种"嵌段交联"型的多功能聚合物的构建展示了一种简单并智能的材料合成方法. 同时, 这种配体介导构建还原敏感型胶束的策略提高了LMW-PEI的转染效率, 为开发新的基因递送系统提供了有价值的思路.

