

Construction of Magnetic Resonance Imaging Visible Polymeric Vector for Efficient Tumor Targeted siRNA Delivery

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Abstract RNA interference (RNAi), known for the highly efficient targeted gene silencing, has been demonstrated to be a promising means for cancer treatment. Meanwhile, an effective approach for siRNA delivery is urgently needed to meet the needs for its clinical application. Herein, we constructed a polymeric vector labeled with superparamagnetic iron oxide (SPIO) for magnetic resonance imaging (MRI) visible siRNA delivery. EGFR antibody was also modified to the surface of nanodrug to enhance the delivery effect. Our results showed that the vector exhibited great siRNA complexation ability and mediated an increased endocytosis of siRNA without obvious cytotoxicity. Besides, both *in vitro* and *in vivo* studies evidenced the vector could effectively deliver siRNA into tumor cells, exert highly interfering effect, and show potent MR imaging capacity. The study provides a promising MRI-visible and EGFR targeting delivery system to improve RNAi efficacy for cancer therapy.

Keywords Polymeric vector; siRNA delivery; Magnetic resonance imaging visible; Tumor targeting

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INTRODUCTION

RNA interference (RNAi), known for the highly efficient targeting gene silence, has drawn great attention since being proposed from 1998.^[1] Numerous studies have provided strong evidences that RNAi possesses great potentials for the treatment of many intractable diseases including cancer,^[2,3] cardiovascular disorders,^[4] and neurological disease.^[5] Recently, RNA-based strategies have also emerged as a promising candidate for the vaccine of various cancers as well as coronavirus-COVID-19.^[6–8] owing to their great preponderance of specificity and high efficiency. However, the efficacy is greatly limited by the rapid enzymatic degradation and poor cellular uptake of RNAs.^[9,10] In this case, effective RNA delivery systems are urgently needed to overcome these limitations at present.

Among all of the reported delivery systems, cationic polymers have been demonstrated as effective carriers for RNA delivery, on account of their high delivery efficiency, less immunogenicity/toxicity, and tailorable structures for modification.^[11,12] Up to now, polymers including polyethylenimine (PEI), poly(L-lysine) (PLL), chitosan, and poly (amido amine)

(PAMAM), are commonly used to deliver siRNA.^[13–15] Additionally, branched PEI with high molecular weight, *e.g.*, bPEI_{25k}, has become the golden standard for *in vitro* RNA delivery.^[16,17] The branched PEI possesses high density of cations with multi-level amines, *i.e.*, primary, secondary and tertiary amines, to show high ability for gene condensation, strong proton buffering at a wide range of pH value, easily decoration of targeting molecule, which facilitate the RNA delivery from multiaspect.^[18–20]

Furthermore, superparamagnetic iron oxide (SPIO), a commonly used magnetic resonance (MR) imaging contrast agent to decrease the T2 signal, can be simultaneously and easily introduced *via* amino exchange effect to achieve a noninvasive imaging and guide a more efficient RNA delivery.^[21,22] Our previously reported studies have proved PEI-based systems to be potential vectors for siRNA leaded cancer treatment.^[23–25] Unfortunately, the cumulative toxicity caused by the non-biodegradable PEI severely hinders its *in vivo* applications even the acute cytotoxicity can be decreased by PEGylation.^[26–28]

Aiming at improving the *in vivo* performance of PEI for gene delivery, a variety of strategies have been proposed in recent years. Our previous studies conjugated liner PEI with low molecule weight to the PLL chains to obtain gene delivery system combing the advantage of biodegradability with strong proton buffering.^[29,30] We also used short line PEI or modified poly(L-aspartic acid) (PAsp) to construct siRNA delivery system, in which the siRNA complexation process must be conducted at pH 5.0 and a cross-linked interlayer was needed

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for gene protection.^[31,32] These vectors, of course, dramatically increase the delivery efficiency as well as decrease the toxicity. However, they are complicated either in polymer synthesis or in nanodrug preparation for further application. Another method using tumor acidity cleavable linkage to conjugate small branched PEI to improve the RNA transfection efficiency, in which the final molecular weight of vector was uncontrollable and the quality of synthesized nanodrug was affected.^[33] Hence, effective RNA delivery polymers for the substitution of bPEI_{25k} to promote the *in vivo* RNAi application are still needed.

Herein, a multifunctional siRNA delivery system based on a poly(ethylene glycol) and poly(aspartic acid) block polymer (mPEG-PAsp) was proposed. To simulate the multilevel amines of branched PEI, diethylenetriamine (DETA) and *N,N*-dimethylethylenediamine (DMA) were grafted onto the side chain of PAsp, yielding the siRNA delivery polymer mPEG-PAsp(DMA/DETA) (abbreviated as PAME). Then, SPIO was labeled to PAME via amino exchange effect, leading to an MRI-visible carrier (PAME-SPIO) for siRNA complexation. Besides, the epidermal growth factor receptor (EGFR) antibody was conjugated to form a targeted nanodrug (EGFR-NP-SPIO) using Mal-PEG-NHS to increase the transfection efficiency (Scheme 1).^[34] Both *in vitro* and *in vivo* studies were conducted to evaluate the siRNA delivery efficiency of the polymeric vector.

EXPERIMENTAL

General Considerations

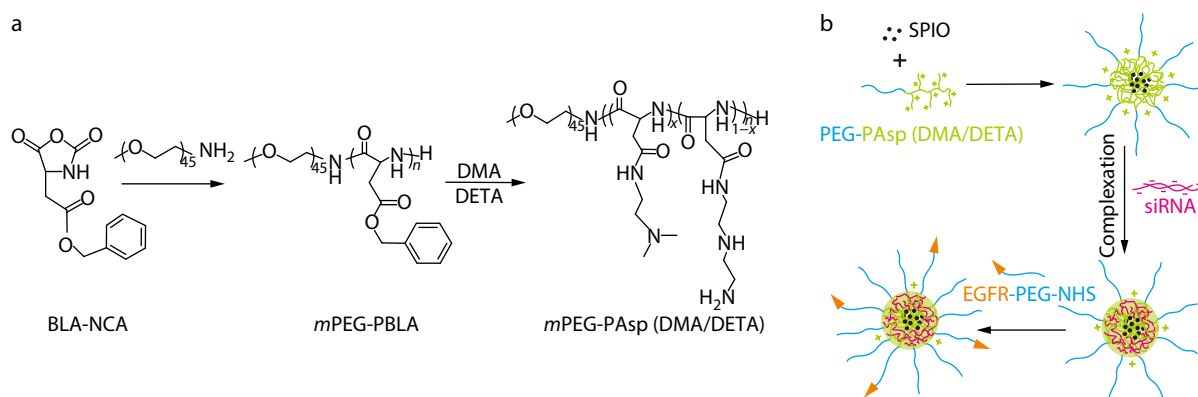
α -Methoxy- ϵ -amino-poly(ethylene glycol) (mPEG-NH₂, $M_n=2$ kDa), anhydrous dimethyl sulfoxide (DMSO) and anhydrous dimethylformamide (DMF) were purchased from Sigma-Aldrich and used as received. Diethylenetriamine (DETA) and *N,N*-dimethylethylenediamine (DMA) were obtained from Alfa Aesar China Co., Ltd. (Tianjin, China). Dialysis bags (MWCO: 3.5 kDa, 14 kDa) were purchased from Shanghai Green Bird Technology Development Co., Ltd., China. The following reagents were of analytical grade and purchased from Guangzhou Chemical Reagent Factory, China: diethyl ether, chloroform (CHCl₃), acetic ether, petroleum ether and dichloromethane (CH₂Cl₂). SPIO was synthesized according to a previous publication.^[35] RPMI-1640 medium, penicillin/streptomycin, fetal bovine serum (FBS) and

Dulbecco's phosphate buffered saline (PBS) were purchased from Gibco BRL (Carlsbad, CA, USA). 4',6-Diamidion-2-phenylindole (DAPI) and Cell count kit-8 were purchased from Beyotime (Beyotime, China). Alexa Fluor 549 was purchased from Molecular Probes (Invitrogen, USA). The FITC-siRNA and siRNA duplexes were purchased from Sangon Biotech (Shanghai, China). The sequence of anti-luciferase siRNA are as follows: 5'-sense strand, 5'-ACAUCACUUACGCU GAGUACUUCGAAG-3' and 5'-anti sense strand, 5'-UCGAAGUACUCA GCGUAAGUGAUGUAU-3'. The EGFR antibody was purchased from Cell Signaling Technology (CST, USA).

Polymer Synthesis and SPIO Labeling

The polymer of PAME was synthesized via two-step reactions outlined in Scheme 1(a) according to our previous study.^[36,37] Firstly, the copolymer of ethylene glycol and β -benzyl L-aspartate (mPEG-PBLA) were synthesized by ring-opening polymerization of BLA-NCA, using PEG-NH₂ as an initiator.^[38] Briefly, 0.50 g of PEG-NH₂ (0.25 mmol) and 4.99 g of BLA-NCA (20 mmol) were dissolved in 60 mL of dry CH₂Cl₂ and 6 mL of anhydrous DMF, and the solution was kept stirring for 72 h at 35 °C followed by precipitated into excessive diethyl ether. The precipitate was filtered, washed with diethyl ether, and vacuum-dried to obtain PEG-PBLA ($M_n=18.4$ kDa, calculated from ¹H-NMR spectrum). Subsequently, ammonolysis of PBLA with DMA and DETA was conducted. 1.84 g of PEG-PBLA (0.1 mmol) and 0.35 g of DMA (4 mmol) were co-dissolved with 20 mL of DMSO and reacted for 24 h at 35 °C. Then, 21 mL of DETA (200 mmol, 50 eq.) was added and the reaction was conducted for another 2 h at 35 °C. The mixture solution was transferred into a dialysis bag (MW cutoff: 7 kDa) and dialyzed against methanol to remove excess DETA and DMSO. The solution was finally precipitated and washed three times by cold diethyl ether to acquire PAME polymer ($M_n=18.8$ kDa, calculated from ¹H-NMR spectrum, total yield: 76%).

SPIO was labeled to PAME by amino exchange reaction.^[23,25] In brief, 50 mg of PAME and 5 mg of SPIO were dissolved in 5 mL of chloroform, and the mixture was then added into 10 mL of water under sonication (VCX130, Sonics, USA, 40% power level) and kept vigorous stirring for 48 h at room temperature. After reaction, the chloroform was removed using rotary evaporation and the solution was filtered through a 450 nm membrane to eliminate free SPIO and large aggregates. Then, the free polymer without SPIO labeling was



Scheme 1 Illustration of polymer synthesis and nanodrug preparation. (a) Synthesis of mPEG-PAsp(DMA/DETA); (b) The preparation of siRNA nanodrug for targeted tumor treatment.

removed by washing samples using ultrafiltration with a MILLIPORE centrifugal filter device (MW cutoff: 50 kDa). Finally, the SPIO labeled polymer (PAME-SPIO) was obtained by filtering the sample through a syringe filter (poresize: 220 nm).

Nanodrug Preparation

PAME-SPIO can encapsulate and concentrate siRNA through electrostatic interaction. Luciferase gene targeting siRNA or scrambled siRNA (SCR) was dissolved into RNase-free water and then mixed with PAME-SPIO according to the designed N/P ratio (the molar ratio of polymer nitrogen to RNA phosphorus). The mixture was vigorously vibrated for 30 s followed by being kept at room temperature for 30 min to form the siRNA or SCR loaded nanoparticles (NP-SPIO). The excess amino groups on the surface of nanoparticles could react with EGFR antibody-PEG-NHS to obtain the final targeted nanodrug (EGFR-NP-SPIO). Concretely, 50 μ L of EGFR antibodies (1 mg/mL) were mixed with 1 mg of tris(2-carboxyethyl)phosphine dissolved in 0.5 mL of PBS (containing 10 μ L of EDTA solution). After incubation at 37 °C for 90 min, the solution was washed with PBS (containing EDTA) using MILLIPORE centrifugal filter device (MW cutoff: 10 kDa) for three times and then incubated with 50 μ g of mal-PEG_{2k}-NHS at 4 °C overnight. Then the obtained EGFR-PEG_{2k}-NHS solution was added into 500 μ L of NP-SPIO (2 mg/mL) and incubated at 4 °C overnight. Then the mixture was washed three times with PBS in MILLIPORE centrifugal filter device (MW cutoff: 10 kDa) to remove the free antibody and tris(2-carboxyethyl)phosphine.

Characterizations of the Polymer and Nanodrugs

¹H-NMR spectra were obtained on a Bruker 400 MHz spectrometer at 25 °C. Fourier transform infrared (FTIR) spectra were recorded on a FTIR spectrometer (Nicolet/Nexus 670, Thermo Electron). The particle sizes and zeta potentials of nanoparticles were measured by dynamic light scattering (DLS). Measurements were performed at 25 °C on Nano-ZS instrument (Malvern Instruments, Malvern, UK). Data were calculated from five times measurements. The morphology of nanoparticles was characterized by transmission electron microscopy (TEM) on a JEOL/JEM-1400 Flash instrument. Samples were prepared by adding a drop (5 μ L, 0.5 mg/mL) of the sample solution on a copper grid coated with amorphous carbon. The grid was dried overnight at 60 °C before TEM observation. The T2 relaxivity of the nanodrug was measured on a clinical 1.5T MR scanner (Siemens, Erlangen, Germany).

Agarose Gel Retardation Assay

Agarose gel electrophoresis assay was conducted to assess the siRNA condensation capacity of the PAME-SPIO. Briefly, siRNA was mixed with PAME-SPIO at various N/P ratios (0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5). Then, the solutions were vortexed for 30 s and incubated at room temperature for another 30 min. After mixing with 50% glycerol, the PAME-SPIO and siRNA complexes were loaded onto a 1% agarose gel with ethidium bromide (0.1 μ g/mL). After gel electrophoresis at 100 V for 40 min, the gel was imaged with a BioRad Gel Doc XR System (BIO-RAD, Hercules, CA, USA).

Cell Culture

Human lung cancer transgenic cell line A549 with constant luciferase gene expression (A549-luc) was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The A549 cells were cultured in

RPML-1640 medium containing 10% FBS, 100 U/mL penicillin/streptomycin and incubated under a humidified atmosphere of 5% CO₂ at 37 °C.

Confocal Laser Scanning Microscope

The A549 cells were seeded on confocal dish at a density of 1×10^6 cells per well and were incubated for 12 h in the incubator. FITC-labeled siRNA (25 nmol/L per well) was used to form FITC labeled NP-SPIO and EGFR-NP-SPIO at the N/P ratio of 2.5. Then, the sample solutions were added to each well. After 10 h of incubation, the medium was discarded and the A549 cells were washed for three times with PBS. After fixed with 4% formaldehyde for 10 min at room temperature, the cells were washed three times with PBS again. Then, the cell nuclei were stained with DAPI for 15 min. The fluorescence of cells was observed and captured with Zeiss LSM 510 META microscope (Carl Zeiss CI, Ltd, Gottingen, Germany). For the free antibody competitive inhibition assay, A549 cells were pre-treated with 10 μ L of free EGFR antibody for 1 h before EGFP-NP-SPIO was added into the culture medium.

Prussian Blue Staining

A549 cells were firstly incubated with different nanoparticles of PAME, NP-SPIO, EGFR-NP-SPIO and EGFR-NP-SPIO + free EGFR, respectively. After 24 h, the medium was removed and cells were washed with PBS for three times and then fixed with 4% of paraformaldehyde for 20 min. After washed again with PBS for three times, Prussian blue staining solution comprised of hydrochloride (3%) and potassium ferrocyanide (1%) was added. After a 60 min incubation at room temperature, cells were washed with PBS for three times and observed under microscope to evaluate the intracellular iron.

Cytotoxicity Assay

To evaluate the cytotoxicity of PAME, MTT assay was performed according to the manufacturer's protocol. A549 cells were seeded in a 96-well plate at a density of 3×10^3 cells per well and then cultured for 12 h at 37 °C. The A549 cells were then incubated for 48 h with 100 μ L of fresh medium at various concentrations of PAME (0, 6.25, 12.5, 25, 50, 100, 150, 200 μ g/mL). Subsequently, the medium was replaced with fresh medium containing MTT solution (5 mg/mL) and incubated for another 4 h. Afterwards, the medium in the wells was discarded and 150 μ L of DMSO was added to each well. After shaking for 8 min, and finally the absorbance value of each well at 490 nm was measured on a Tecan Infinite F200 Multimode plate reader (Tecan Trading AG, Switzerland).

Luciferase Activity Assay *in vitro*

A549-luc cells were seeded in a 96-well plate at a density of 5×10^4 cells per well and then cultured for 12 h at 37 °C. The cells were then treated with different nanodrugs complexing anti-luciferase siRNA at a final siRNA concentration of 50 nmol/L. A nanodrug of EGFR-NP-PEI, using PEG_{2k} modified branched PEI_{25k}^[24] instead of PAME to load SPIO, complex siRNA and conjugate EGFR antibody, was introduced as control. 4 h After incubation, the solutions were replaced with fresh medium (RPML-1640 containing 10% FBS) and cells were then cultured for another 12 h. Then, 20 μ L of lysis buffer and luciferin substrate were added to the wells. Then the suspension was transferred to 96-well and was vortexed for 3 min at room temperature after 100 μ L of luciferase detection agent was

added into each well. The luciferase activity was analyzed using the Pica Gene luciferase assay system (Toyo Ink, Tokyo, Japan). The total-protein was extracted and determined according to the BCA Protein Assay Kit.

Animal Model

Animals were purchased from the Experimental Animal Center of Guangdong Province, and animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jinan University and approved by the Animal Ethics Committee of Jinan University. All experiments involving animals strictly followed the Animal Management Regulations of China (1988, revised 2017) and the Guideline on the Humane Treatment of Laboratory Animals of China (MOST 2006). To establish subcutaneous tumor model, A549-luc cells (5×10^6 in 100 μL of PBS) were implanted subcutaneously into the foreleg of nude BALB/c mice. Tumor volume in subcutaneous tumor model was measured using a caliper and calculated as follows: Tumor volume = $0.5 \times \text{length} \times \text{width}^2$.

In vivo Fluorescence Imaging

When the tumor volume reached about 100 mm^3 , the nude mice were injected with NP-SPIO and EGFR-NP-SPIO complexing AF750-labeled SCR (SCR dose: 300 $\mu\text{g}/\text{kg}$ body weight) *via* tail vein, respectively. The mice were then anesthetized, and the fluorescence images were taken using a live fluorescence imaging system instrument (Bruker, Germany). The excitation and emission wavelengths chosen for the AF750 were 720 and 790 nm, respectively, and the exposure time was 30 s.

In vivo Magnetic Resonance Imaging

MRI was recorded after the injection of NP-SPIO and EGFP-NP-SPIO with a dose of 2 mg Fe/kg body weight using a 1.5 T MRI scanner (Siemens, Erlangen, Germany). T2-weighted images were acquired using a fast spin-echo sequence with the following set of parameters: TR=1600 ms, TE=30 ms, and NSA=2, field of view: 4 cm $\times$ 4 cm; slice thickness 1.0 mm; slices: 15; interslice distance: 1 mm; repetition time: 3 s; flip angle 90°. The signal intensity of tumors after injection was normalized by comparison with that of tumors before injection and calculated with the software tools provided by the manufacturer.

In vivo Luciferase Interference

Nanodrugs of NP-SPIO and EGFR-NP-SPIO complexing anti-

luciferase siRNA were formed as already described. In addition, EGFR-NP-SPIO complexing SCR (SCR group) was used as control. When the tumor volume reached about 100 mm^3 , the mice were divided into three groups, and were intravenously injected with SCR, NP-SPIO, EGFR-NP-SPIO through tail vein at 0 h, 24 h and 48 h with a siRNA dose of 300 $\mu\text{g}/\text{kg}$ body weight, respectively. The mice were anesthetized 5 min after intraperitoneal injection of Luciferin (100 μL), and the bioluminescence images were captured using a live fluorescence imaging system instrument (Bruker, Germany) at various time points.

Statistical Analysis

Data were statistically analyzed using Prism 6.0 software (GraphPad, San Diego, CA, USA). The results were presented as the mean $\pm$ standard deviation (SD), and $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Preparation and Characterization of Polymer and Nanodrugs

The successful synthesis of polymers *via* two-step reactions was confirmed by ^1H -NMR and FTIR analyses. The *m*PEG-PBLA was firstly synthesized by ring-opening polymerization of BLA-NCA with *m*PEG-NH₂ used as initiator. As shown in Fig. 1(A), the major resonance peaks of the copolymer PEG-PBLA in the ^1H spectrum well fit into the expected chemical structure as follows: 3.42–3.56 ppm (m, $-\text{OCH}_2\text{CH}_2-$ of PEG, a), 4.55–4.69 ppm (m, $-\text{NH}(\text{CH}_2)\text{CHCO}-$ of PBLA, b), 2.54–2.92 ppm (m, $-\text{CHCH}_2\text{CON}-$ of PBLA, c), 4.92–5.12 ppm (m, $-\text{CH}_2\text{C}_6\text{H}_5$ of PBLA, d), 7.15–7.40 ppm (m, $-\text{CH}_2\text{C}_6\text{H}_5$ of PBLA, e), 8.10–8.25 ppm (m, $-\text{CHCH}_2\text{CONH}-$ of PBLA, f). The average polymerization degree of PBLA was 78 according to the integral area ratio of proton from methylene group in PEG and benzyl group in PBLA. After ammonolysis, the characteristic peaks of benzyl group (4.92–5.12 ppm, d, and 7.22–7.42 ppm, e) disappeared and the characteristic peaks of DMA (2.75–2.95, i; 3.15–3.40 ppm, h) and DETA (2.45–2.75 ppm, g) appeared in ^1H spectrum of *m*PEG-PAsp(DMA/DETA) (Fig. 1B). The complete ammonolysis reaction was further verified by FTIR analysis, exhibiting the disappearance of the prominent ester absorption around 1740 cm^{-1} and the benzene vibration absorption around 745 and 696 cm^{-1} (Fig. 2), which was attributed to the

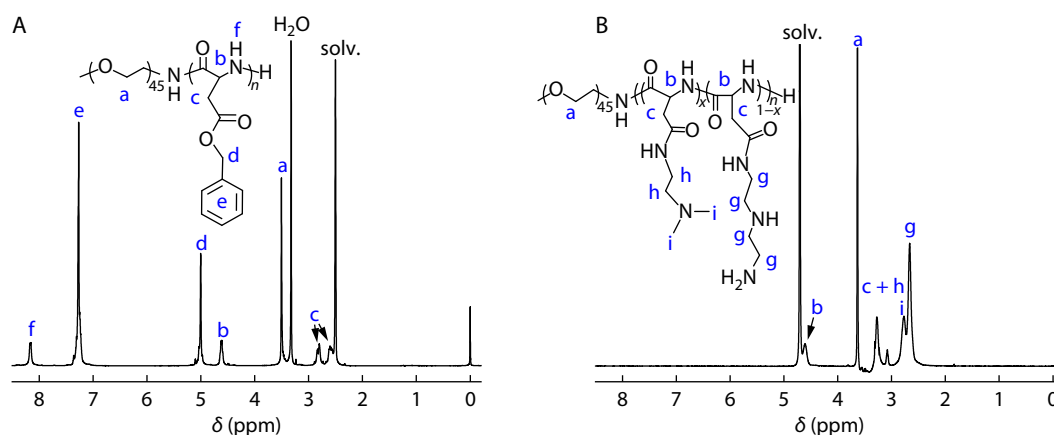


Fig. 1 ^1H -NMR spectra of (A) *m*PEG-PBLA and (B) *m*PEG-PAsp(DMA/DETA) in DMSO- d_6 and $\text{D}_2\text{O}-\text{d}_2$, respectively.

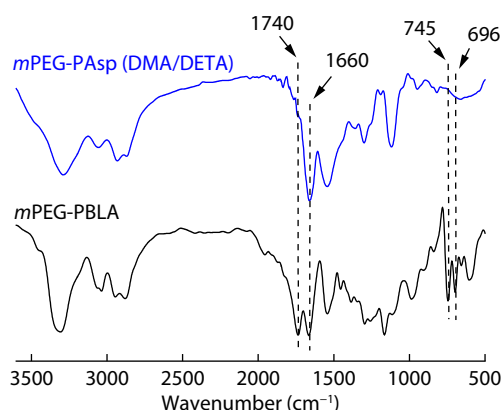


Fig. 2 FTIR spectra of mPEG-PBLA and mPEG-PAsp(DMA/DETA).

deprotection of benzyloxycarbonyl groups. These results evidenced the successful synthesis of polymer. Subsequently, agarose gel electrophoresis was employed to investigate the capacity of PAME-SPIO to complex siRNA at various N/P ratios. The complexation of siRNA with polymers was due to the electrostatic interaction, which further resulted in the retardation of siRNA mobility in the electric field. As shown in Fig. 3(a), the mobility of siRNA is gradually retarded with the increase of N/P ratio, and was completely retarded when the N/P ratio reached 2.0. The gel electrophoresis assay demonstrated that the synthesized PAME-SPIO showed more excellent siRNA complexation even compared with the branched PEI with high molecular weight,^[39] which was the essential property for gene delivery system. To obtain

complexes with enough amino groups for antibody modification, the nanodrug formed at an N/P ratio of 2.5:1 was selected for subsequent experiments. The particle size and zeta potential of nanodrugs were detected by DLS measurements, finding that the size of NP-SPIO increases from 38.4 ± 12.1 nm to 75.9 ± 4.4 nm after PAME-SPIO complexed siRNA, whereas the zeta potentials significantly decrease from 28.52 ± 0.75 mV to 14.3 ± 2.15 mV. The positively charged potential implied a complete complexation and well protection of siRNA. After the targeted EGFR antibody was modified to the surface, EGFR-NP-SPIO shows a particle size of 76.7 ± 3.8 nm and a still positively charged zeta potential of 8 ± 1.44 mV (Fig. 3b), which were suitable for long time blood circulation and effective cellular uptake.^[40] TEM images of EGFR-NP-SPIO show spherical morphology with a uniform size around 70 nm, which was in line with the DLS detection results. In addition, SPIO can be observed clearly accumulated in the nanoparticles (Fig. 3c). The cluster of SPIO is expected to result in an enhanced T2 relaxivity for better magnetic resonance imaging.^[41] As shown in Fig. 3(d), the T2 relaxivities of NP-SPIO and EGFR-NP-SPIO are 0.3595×10^6 and 0.3776×10^6 $\text{M}^{-1} \cdot \text{s}^{-1}$, respectively, which was obviously higher than that of PAME-SPIO (0.2270×10^6 $\text{M}^{-1} \cdot \text{s}^{-1}$). These results indicated that our polymeric system could effectively complex siRNA to form nanoparticles with high T2 relaxivity, leading to an MRI-visible and highly efficient siRNA delivery.

Cytotoxicity and *in vitro* Cellular Uptake

The cytotoxicity of PAME was evaluated by MTT assay. As shown in Fig. 4(a), with the gradually increased concentration of PAME to 200 $\mu\text{g}/\text{mL}$, more than 80% of A549 cells survived. This result illustrated that PAME showed a negligible cytotoxicity

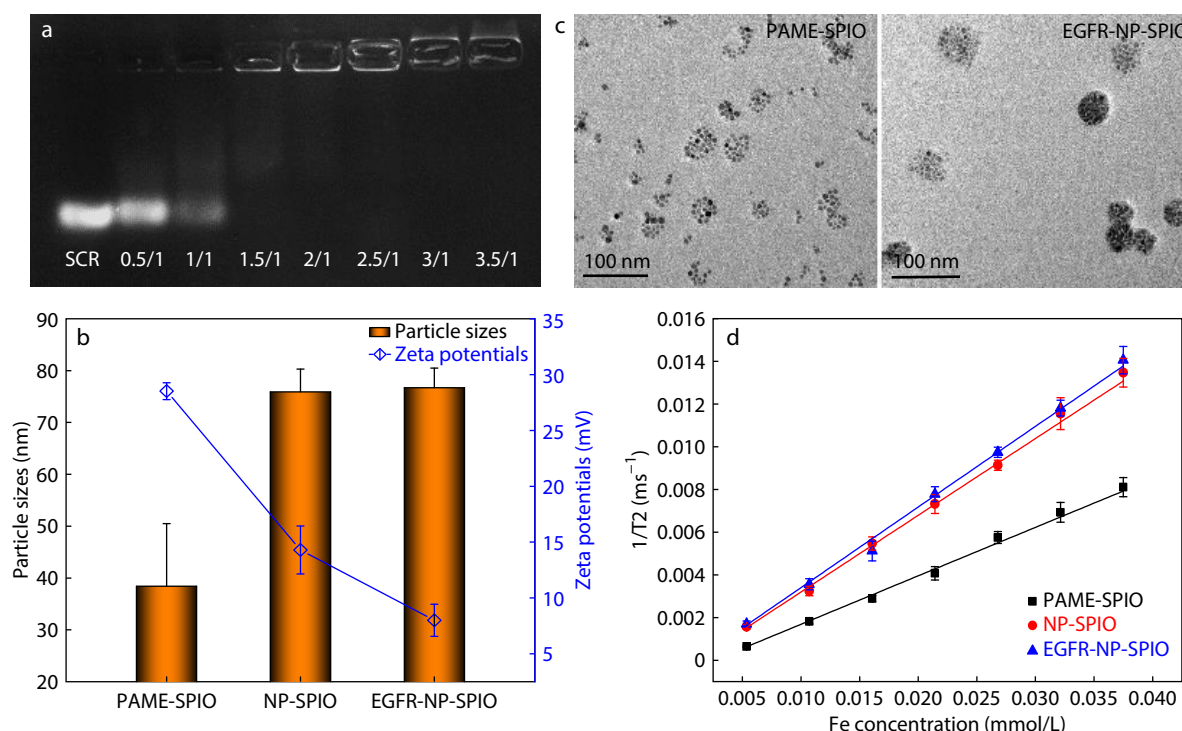


Fig. 3 Characterization of MRI-visible siRNA nanodrugs. (a) Electrophoretic mobility of siRNA in agarose gel after complexing with PAME-SPIO at various N/P ratios; (b) Particle sizes and zeta potentials of PAME-SPIO, NP-SPIO, EGFR-NP-SPIO nanoparticles; (c) TEM images of PAME-SPIO and EGFR-NP-SPIO; (d) The T2 relaxivities of PAME-SPIO, NP-SPIO, and EGFR-NP-SPIO were 0.2270×10^6 , 0.3595×10^6 and 0.3776×10^6 $\text{M}^{-1} \cdot \text{s}^{-1}$, respectively.

compared to the branched PEI_{25k} as reported.^[32] To investigate the cellular uptake of nanodrugs by A549 cells, fluorescence imaging and Prussian blue staining were carried out. As shown in Fig. 4(b), FITC-labeled siRNA was used to visualize the cell uptake of siRNA. After incubation with the NP-SPIO, the green fluorescence (siRNA) can be observed in A549 cells, demonstrating that siRNA was successfully delivered into A549 cells. Besides, much stronger green fluorescence is observed in the targeting group of EGFR-NP-SPIO, indicating that EGFR modification enhanced the cellular uptake of nanodrugs. To further demonstrate that the increased uptake of nanodrugs was mediated by EGFR, we added an excess of free EGFR antibody together with EGFR-NP-SPIO. We found that the FITC fluorescence intensity decreased after free EGFR antibody co-incubation, indicating that EGFR targeting could promote the uptake of nanodrugs by A549 cells. Similarly, Prussian blue staining showed abundant iron inside the transfected cells, indicating that SPIO was successfully delivered into the A549 cells, and the enhanced cell uptake of targeted nanodrug was mediated by EGFR (Fig. 4c). These results indicated our polymeric system could effectively deliver siRNA into A549 cells without obvious cytotoxicity, implying the PAME to be a promising RNA delivery vector.

In vitro Gene Silencing Effect of siRNA Nanodrugs

As siRNA was effectively delivered into tumor cells by the polymeric vector, whether it could silence the targeted gene became our concern. The firefly luciferase-expressing A549 cells

were treated with nanodrugs bearing siRNA targeting luciferase gene. Then the luciferase protein was detected after nanodrug treatment to verify the gene silencing effect *in vitro*. As shown in Fig. 5, compared with control group, the luciferase expression in NP-SPIO treatment group was moderately suppressed. Contrastively, the luminescence efficiency of A549 cells treated with EGFR-NP-SPIO is found to be significantly decreased compared to those treated with NP-SPIO group. Additionally, the therapeutic effect was impeded by the co-incubation of free EGFR antibody since the high cell uptake efficiency of targeted nanodrug was inhibited. Notably, the *in vitro* gene silencing effect of EGFR-NP-SPIO is comparable to that of EGFR-NP-PEI nanodrug formed by the commonly used transfection reagent bPEI (Fig. 5). These results demonstrated that the EGFR targeting delivery system can effectively deliver siRNA into A549 cancer cells and exert highly efficient gene silencing effect.

In vivo Tumor Accumulation of Nanodrugs

In order to validate the tumor-targeted siRNA delivery capacity of the polymer *in vivo*, a subcutaneous tumor model in nude mice was successfully established. Fluoresce visible NP-SPIO and EGFR-NP-SPIO nanoparticles were prepared by complexing AF750-labeled SCR. As shown in Fig. 6(a), an obvious tumor accumulation effect can be detected in both NP-SPIO and EGFR-NP-SPIO treatment groups. The fluorescence intensity in the tumor site reached the highest at 3 h post injection and gradually decreased due to the clearance of nanoparticles.

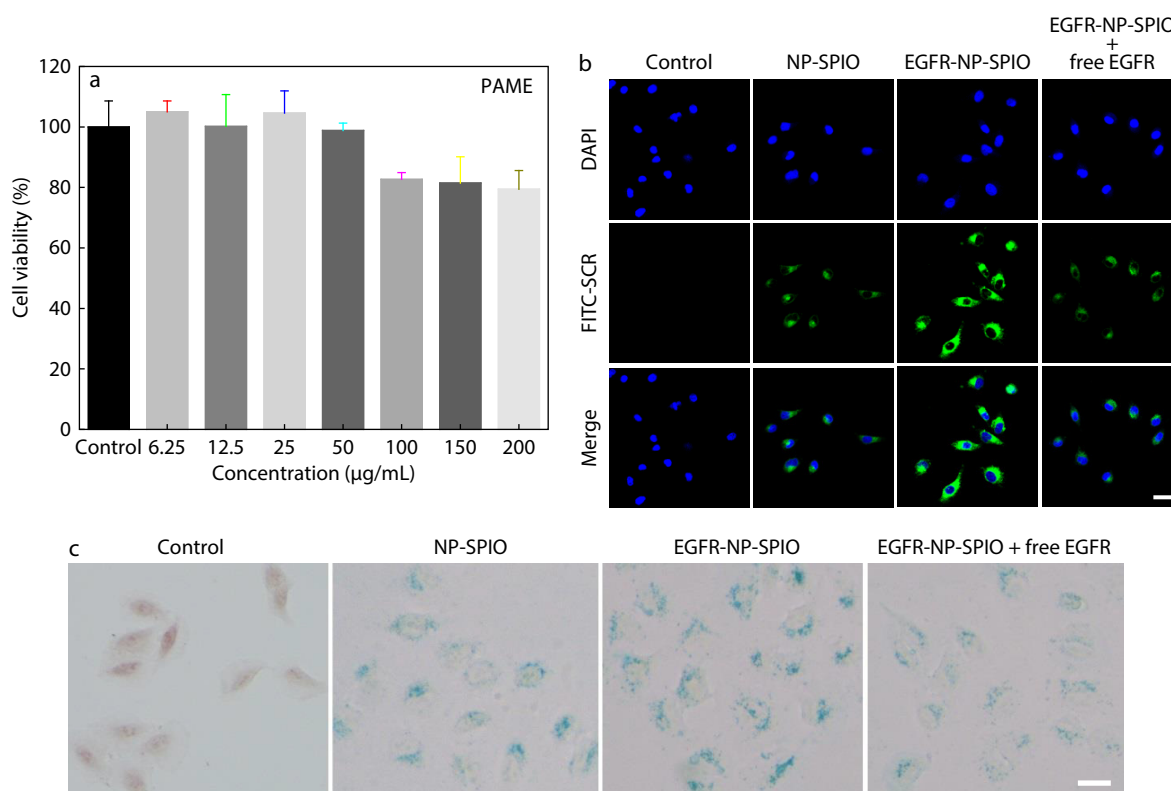


Fig. 4 Cytotoxicity and *in vitro* cellular uptake of nanodrugs. (a) Cell viability of A549 cells after incubation with PAME at different concentrations (0, 6.25, 12.5, 25, 50, 100, 150, 200 µg/mL) for 48 h from MTT detection; (b) Laser confocal microscopic images of A549 cells after incubation with SCR, NP-SPIO, EGFR-NP-SPIO and EGFR-NP-SPIO plus free EGFR, respectively. The siRNA was labeled by FITC. Incubation time: 4 h, scale bar: 25 µm; (c) Prussian blue staining for SPIO distribution inside A549 cells after incubation with different nanoparticles for 6 h. Scale bar: 25 µm.

Compared with NP-SPIO group, EGFR-NP-SPIO group shows a significant higher AF750 fluoresce in the tumor site, indicating siRNA in targeted group was delivered more efficiently into

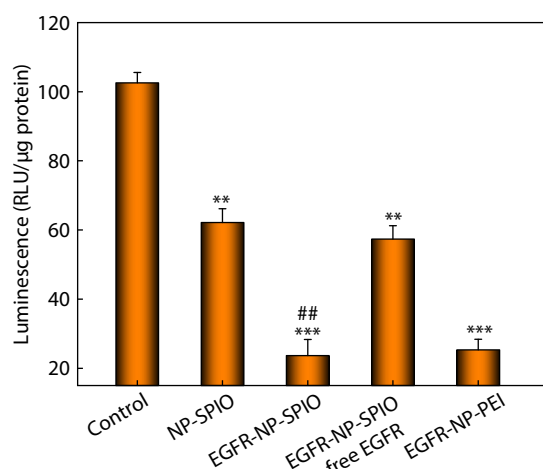


Fig. 5 Gene silencing effect of nanodrugs *in vitro*. Luciferase expression in firefly luciferase-expressing A549 cells treated with nanodrugs bearing siRNA targeting luciferase gene *in vitro*. Dose: 50 nmol/L siRNA. Data are mean±SD ($n=3$). ** $P<0.01$, *** $P<0.001$ compared with "Control" group. ## $P<0.01$ compared with the "NP-SPIO" group.

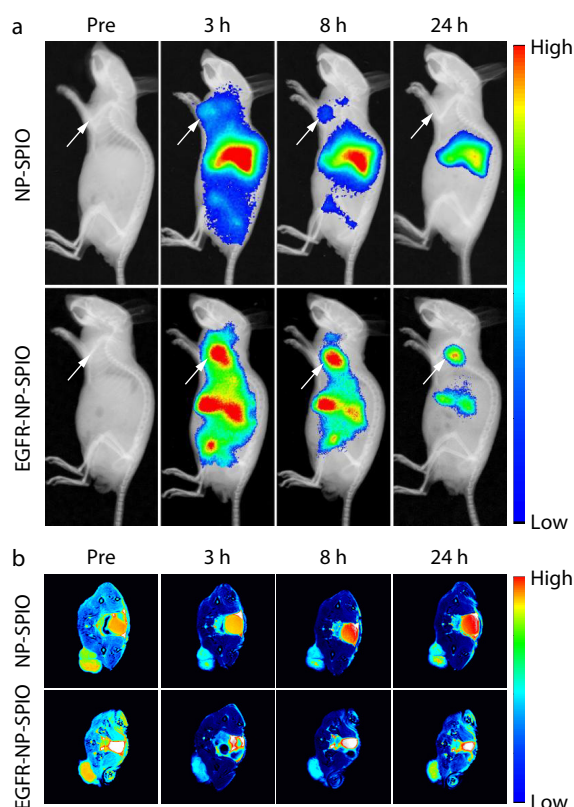


Fig. 6 *In vivo* tumor accumulation of nanodrugs. (a) *In vivo* fluorescence images of tumor-bearing mice injected with AF750 labeled NP-SPIO and EGFR-NP-SPIO *via* tail vein. The white arrow indicates the location of the tumor. siRNA dose: 300 μg/kg body weight. (b) T2-weighted images of mice at different time points after sample injection.

tumor. Moreover, the *in vivo* tumor targeting capacity of the vector was also verified by MRI. Mice were injected with targeting and nontargeting nanoparticle solution through tail vein at a dose of 2 mg Fe/kg body weight, and then the tumor targeted delivery were assessed on a 1.5T MRI scanner. As shown in Fig. 6(b), in line with the fluorescence imaging results, the tumor shows a clear darkening effect and a decrease of T2-weighted signal intensity in the EGFR-NP-SPIO group than that of the nontargeted one. Together, our results showed that EGFR-NP-SPIO could actively target tumor to effectively deliver siRNA into tumor cells *in vivo*, and the SPIO labeling endowed the vector an excellent MRI-visible capacity to guide the delivery process.

***In vivo* Gene Silencing Effect of Nanodrugs**

Finally, to assess the gene silencing effect *in vivo*, mice bearing lung cancer implanted by A549-luc cells receiving the treatment of nanodrugs were subjected to luminescence imaging. The change of luciferase activity inside each group reflects the gene silencing ability of the nanodrugs. Therefore, the bioluminescence intensities relative to the basic level was analyzed. As shown in Fig. 7, in the SCR treatment group, a constantly increased luciferase expression can be detected. Compared to the basic gene expression, no appreciable luciferase gene knockdown was shown in NP-SPIO treatment group even the luciferase expression did not increase with time. However, the animal clearly shows a gradual decrease of luciferase fluorescence intensity after receiving the treatment of EGFR-NP-

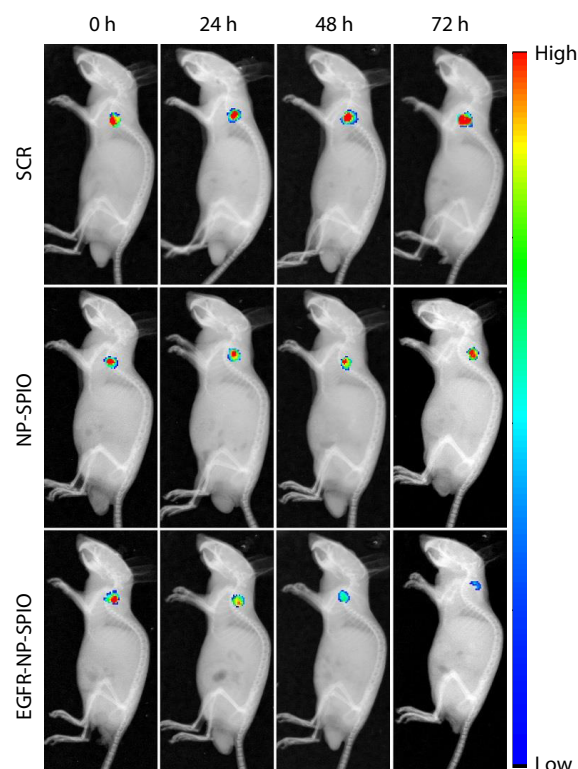


Fig. 7 Gene silencing effect of nanodrugs *in vivo*. Luminescent images of tumor site in tumor-bearing mice after nanodrug treatment. Nanodrugs were injected *via* tail vein at 0, 24 and 48 h with a siRNA dose of 300 μg/kg body weight per injection. SCR group was prepared using EGFR-NP-SPIO complexed with scramble siRNA.

SPIO, indicating luciferase gene was effectively silenced by the deliver siRNA. These results suggested that siRNA delivered by the EGFR-NP-SPIO exerted desirable gene silencing effect *in vivo*, demonstrating the polymer a potential vector for tumor targeted siRNA delivery.

CONCLUSIONS

In conclusion, a block polymer consisting of PEG and PAsp(DMA/DETA) was synthesized for siRNA delivery. The polymer forms by biodegradable polyamino acid main chain modified with multilevel amines to exert enhanced siRNA complexation and reduced toxicity. In addition, targeting molecule of EGFR antibody can be expediently conjugated on to the surface of the nanodrug, resulting in a targeted siRNA delivery system. Furthermore, the polymeric vector is easily labeled by the contrast agent of SPIO, resulting in an MR-visible carrier to guide and facilitate the siRNA delivery. Due to these distinct features, highly effective siRNA delivery and targeted gene silencing were achieved both in cell and animal tests, which revealed the great potential of our siRNA nanodrug for tumor therapy.

NOTES

The authors declare no competing financial interest.

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