Electronic Supplementary Information (ESI)

Construction of negatively charged and environment-sensitive nanomedicine for tumor-targeted efficient siRNA delivery

Yong Wang,[a†] Hong Xiao,[a†] Jing Fang,[b] Xingsu Yu,[b] Zhenwei Su,[a] Du Cheng,[a] Xintao Shuai[*a,b]

[†]These authors contributed equally to this work.

[a]School of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou 510275, China

[b]Center of Biomedical Engineering, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China

*Correspondence should be addressed to:

Xintao Shuai

Tel.: +86-20-84110365

Fax: +86-20-84112245;

E-mail: shuaixt@mail.sysu.edu.cn

Materials

The following reagents were purchased from Sigma-Aldrich and used as received: α-Methoxy-ε-hydroxy-poly(ethylene glycol) (mPEG-OH, Mn = 2 kDa), paratoluensulfonyl chloride (TsCl), succinic anhydride (SA), N,N-Diisopropylamino ethylamine (DIP), captoethylamine (MEA), glutathione (GSH), dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), folic acid (FA), dimethyl sulfoxide (DMSO), dimethylformamide

(DMF). Dialysis bag (MWCO: 3.5 kDa) was purchased from Shanghai Green Bird Technology Development Co., Ltd., China. Tetrahydrofuran (THF) was pre-dried with alloy of potassium and sodium, Chloroform ($CHCl_3$), acetic ether, petroleum ether, and dichloromethane (DCM, CH_2Cl_2) were dried over CaH_2 and then distilled under ambient pressure. Diethyl ether was of analytical grade and purchased from Guangzhou Chemical Reagent Factory, China. mPEG-NH_2 was synthesized from mPEG-OH according to the literature.[1] N-carboxyanhydride of β-benzyl-L-aspartate (BLAsp-NCA) was synthesized as previously reported.[2]

Cell culture medium, trypsin-EDTA and fetal bovine serum (FBS) were purchased from Life Technologies Corporation. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Cell counting Kit-8 (CCK-8) was purchased from Dojindo. Human hepatocellular carcinoma cell line Bel-7402 was purchased from Chinese Academy of Sciences. The fluorescent reagents DAPI, FITC labeled siRNA, and AF750-NHS-ester for siRNA labeling were purchased from Beyotime (Haimen, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Sun Yat-sen University. Female nude mice BALB/c were ordered from Vital River Laboratories (Beijing, china).

Synthesis of mPEG-PBLA

Poly(ethylene glycol)-block-poly(β-benzyl *L*-aspartate), i.e. mPEG-PBLA, was synthesized by ring-opening polymerization of N-carboxy anhydride of β-benzyl *L*-aspartate (BLA-NCA) with mPEG-NH_2 as a macroinitiator.[2] In brief, 2.0 g of PEG-NH_2 (1 mmol) was vacuum-dried at 70 °C for 4 h in a 100 mL flask, and then dissolved in 60 mL of anhydrous dichloromethane. Subsequently, 1.5 g BLAsp-NCA (6 mmol) dissolved in 6 mL of anhydrous DMF was added into the above solution under the protection of argon. The reaction was kept stirring for 72 h at 35 °C. Then, the mixture was precipitated into excessive cool diethyl ether, filtered, washed with diethyl ether, and vacuum-dried until a constant weight was attained (mPEG-PBLA: Mn = 3.2 kDa, calculated from ^{1}H NMR spectrum; yield: 96%).

Synthesis of mPEG-PBLA-COOH

mPEG-PBLA-COOH was obtained by the reaction of mPEG-PBLA-NH_2 with succinic

anhydride. Briefly, mPEG-PBLA-NH_2 (3.0 g, 0.94 mmol), succinic anhydride (0.94 g, 9.4 mmol), and anhydrous $CHCl_3$ (50 mL) were charged into a flask equipped with a stirring bar and a reflux condenser. The flask was closed, placed in an oil bath thermostated at 70 °C, and then the solution was stirred for 72 h. The reaction mixture was precipitated 2 times into a large amount of cool ethanol to remove excessive succinic anhydride, filtered, washed with deionized water and freeze-dried to obtain purified mPEG-PBLA-COOH (Mn = 3.3 kDa, calculated from ^{1}H NMR spectrum; yield: 89%).

Synthesis of BA-PAsp(DIP)

N-butylamine-termined poly(β-benzyl L-aspartate) (BA-PBLA) was first synthesized by ring-opening polymerization of BLA-NCA using n-butylamine as an initiator. Similar to the synthesis of mPEG-PBLA, BLA-NCA (4.48 g, 18 mmol) reacted in the presence of n-butylamine (0.60 mmol, 0.74 g/mL). Next, ammonolysis reaction was carried out according to the literature.[3] 3.5 g of BA-PBLA (0.57 mmol) and 10 mL of *N, N*-diisopropyl ethylenediamine (80 mmol, about 5 eq.) were dissolved in 30 mL of anhydrous DMSO, and then the solution was stirred for 24 h at 35 °C. After the reaction, the solution was dialyzed (MWCO: 3.5 kDa) against methanol for 48 h to remove excess DIP, distilled using rotary evaporation, and finally vacuum-dried to get the PAsp(DIP) (Mn = 7.1 kDa, calculated from ^{1}H NMR spectrum; yield: 86%).

Synthesis of mPEG-PBLA-PAsp(DIP)

mPEG-PBLA-PAsp(DIP) was synthesized by amidation of PEG-PBLA-COOH with PAsp(DIP). Briefly, 0.6 g of mPEG-PBLA-COOH (0.18 mmol), 27 mg of N-hydroxysuccinimide (NHS, 0.23 mmol) and 45 mg of EDC•HCl (0.23 mmol) were dissolved in 30 mL anhydrous DMSO, and the solution was stirred for 30 min to activate the carboxyl group. After 1.47 g PAsp(DIP) (0.2 mmol) was added to the solution, the reaction proceeded for 24 h at room temperature. Subsequently, the mixture solution was precipitated into cool ethyl alcohol, filtered, washed withdeionized water (pH 5.0) and freeze-dried to obtain purified mPEG-PBLA-PAsp(DIP) (Mn = 10.3 kDa, calculated from ^{1}H NMR spectrum; yield: 84%).

Synthesis of mPEG-b-PAsp(MEA)-b-PAsp(DIP)

mPEG-PAsp(MEA)-PAsp(DIP) was synthesized by aminolysis of MEA with mPEG-PBLA-PAsp(DIP).[2] Briefly, 1.0 g of mPEG-PBLA-PAsp(DIP) (0.1 mmol) and 0.92 g of MEA (12 mmol, about 20 eq.) were dissolved in 30 mL of anhydrous DMSO, and then the reaction was stirred for 48 h at 35 °C. The mixture was dialyzed against deionized water for 3 days and then freeze-dried to obtain mPEG-PAsp(MEA)-PAsp(DIP) (Mn = 10.2 kDa, calculated from ^{1}H NMR spectrum; yield: 93%).

Synthesis of FA-PEG-b-PAsp(MEA)-b-PAsp(DIP)

The folate-terminated copolymer was synthesized as previously reported.[1] In brief, α-allyl-ε-hydroxyl-poly(ethylene glycol) (Allyl-PEG-OH, Mn=2000) was first synthesized by anionic polymerization of ethylene oxide as described in the literature.[4] Afterwards, allyl-PEG-PAsp(MEA)-PAsp(DIP) was synthesized by the same method for synthesizing PEG-PAsp(MEA)-PAsp(DIP), and then converted to NH_2-PEG-PAsp(MEA)-PAsp(DIP) by addition reaction of double bonds with cysteamine using AIBN as initiator. Finally, folate was conjugated to the polymer to produce FA-PEG-PAsp(MEA)-PAsp(DIP) using the aforementioned amidation reaction (Scheme S2).

Characterizations

^{1}H NMR spectra were carried out on a Varian Unity 300 MHz spectrometer using DMSO-d_6 as a solvent. FTIR spectral measurements were recorded using a Nicolet/Nexus 670 FTIR spectrometer with a resolution of 2 cm^{-1} and the powder samples were compressed into KBr pellets. The molecular weight distribution of copolymer was analyzed using a gel permeation chromatography (GPC) system consisting of a Waters 1515 pump, an Ultrahydrogel TM 500 column, an Ultrahydrogel TM 250 column, and a Waters 2417 differential refractive index detector with PEG as a calibration standard. THF was used as an eluent at a flow rate of 1.0 mL/min. The fluorescence spectra were measured on a spectrofluorophotometer (Perkin Elmer Ltd., United Kingdom). Raman spectroscopy was measured using a FT-Raman Spectrometer (Nicolet NXR 9650, USA) with excitation wavelength of 1064 nm. The sulphydryl contents of the non-crosslinked and crosslinked polyplexes were determined by Ellman's reagent (DTNB) as reported before.[2]

Transmission electron microscopy (TEM) was performed using a Hitachi model H-7650 TEM

operated at 80 kV. The samples were prepared by drying a drop (10 μL, 1 mg/mL) of the sample solution on a copper grid coated with amorphous carbon. For the negative staining of samples, a small drop of uranyl acetate solution (2 wt% in water) was added to the copper grid, which was then blotted with a filter paper after 1 min. The grid was finally dried overnight in a desiccator before TEM observation.

The sizes and zeta potentials of polyplexes of PEG-PAsp(MEA)-PAsp(DIP) and siRNA were determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C on a 90 Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA). For the zeta potential measurement, a standard electrophoresis mini-cell from Brookhaven was used. The data for particle size and zeta potential were collected on an auto-correlator with a detection angle of scattered light at 90° and 15°, respectively. For each sample, the data from five measurements were averaged to obtain the mean ± standard deviation (SD).

Preparation of nanopolyplexes (NP)

After the triblock copolymer was dissolved in phosphate buffer saline(PBS, pH 5.0), the siRNA (dissolved in sterile water) was added into the polymer solution at predetermined amounts according to N/P ratios. The mixture was vigorously vibrated for 30 seconds and kept at room temperature for 30 minutes to form polyplex. Subsequently, the interlayer crosslinking of polyplex by disulfide linkage was achieved by bubbling of an oxygen flow for 30 minutes. Finally, the solution was adjusted to pH 7.4 for other experiments.

Determination of siRNA complexation via gel retardation assay

Complexation of the polymer with siRNA to form polyplexes was evaluated by agarose gel electrophoresis. The experiment was conducted at pH 5.0 and the polyplexes were prepared as aforementioned without crosslinking the interlayer and adjusting pH value. Polyplexes were loaded onto 1% agarose gels with ethidium bromide (0.5 mg/mL), and the electrophoresis was performed in 1×TAE buffer at 100 V for 25 min on a Bio-Rad Sub-Cell electrophoresis cell (Bio-Rad Laboratories, Inc., US). Images were obtained on a DNR Bio-Imaging Systems (DNR Bio-Imaging Systems Ltd., Israel). The retardation of siRNA mobility was detected by irradiation with UV light.

Detection of serum stability of polyplexes

To evaluate the serum stability of polyplexes used in the biological tests, polyplexes were incubated at 37 °C in PBS supplemented with 10% FBS. The change of polyplex size over time was monitored up to 24 h using a 90 Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA).

Dual sensitivity evaluated by fluorometric assay

The FA-NPwas prepared by complexing FITC-SCR with FA-targeted polymer at the N/P ratio of 6. The polyplex solutions were grouped as follows: (1) pH 5.0 + 10 mM GSH, (2) pH 7.4, (3) pH 7.4 + 5 μM GSH, (4) pH 7.4 + 10 mM GSH. The FITC-SCR concentration for each group was 2 μg/mL. The FITC fluorescence emission of each solution from 498 nm to 600 nm was measured with an excitation wavelength of 488 nm on a RF5301PC spectrophotometer (Shimadzu, Japan) at predetermined time points. In the case of dual stimulations, GSH was added before pH adjusting.

Cell viability assay

Human hepatic cancer Bel-7402 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and then cultured for 24 h in 100 μL of RMPI-1640 (Gibco) containing 10% FBS (Gibco) and 1% antibiotics (penn/strep, Invitrogen Corporation) in a humidified atmosphere with 5% CO_2. Subsequently, the cells were incubated with polymer or polyplex (N-NP, FA-NP, P-NP, PEI-NP) for 24 h at concentrations ranging from 50 to 500 μg/mL in the same medium. After 24 h co-incubation, the old medium of each well was replaced with 100 μL fresh medium containing 10 μL MTT solution (5 mg/mL in PBS) and incubated for 4 h at 37 °C. Then 100 μL DMSO was added to dissolve the formazan after discarding the supernatant. After vibration for 15 min at room temperature, the absorbance at 570 nm of each well was recorded using a Tecan Infinite F200 Multimode plate reader. All experiments were conducted in triplicate.

Laser scanning confocal microscopy (LSCM)

The intracellular distribution of FITC-labeled siRNA was detected using LSCM. Bel-7402 cells were seeded in a confocal dish at a density of 1×10^3 cells per dish and incubated overnight in RPMI-1640 containing 10% FBS. After incubation with different polyplexes for 8 hours, the cells

were washed three times with PBS, and then the nuclei were stained with DAPI(Beyotime Biotech, China) for 20 min in order to identify the FITC-SCR location. In addition, free FITC-SCR was introduced as control. The cells were observed under a ZeissLSM710 microscope (Zeiss, Germany). The excitation wavelengths of FITC and DAPI were 488 and 352 nm, respectively.

Flow cytometry analysis

Cells incubated with polyplexes were trypsinized, washed and re-suspended in 0.5 mL of PBS, and finally measured by flow cytometry using a 488 nm laser for excitation. The fluorescence emission ofFITC was recorded at 525 nm. Normally cultured cells without transfection were measured for calibrating the background.

In vitro RNA interference efficiency evaluation

Bel-7402 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated overnight in 100 μL of RPMI-1640 containing 10% FBS. Transfection experiment was performed with 0.2 μg of luciferase DNA per well using Lipofectamine2000 as vehicle in serum-free RPMI-1640. After cells were incubated at 37 °C for 6 hours in a humidified atmosphere with 5% CO_2, transfection solution was replaced with fresh RPMI-1640 containing 10% FBS and the cells were incubated for an additional 12 hours at 37 °C. Then polyplexes loaded with luciferase siRNA in 100 μL serum-free RPMI-1640 were added to each well. Afterwards, the media was replaced with fresh RPMI-1640 containing 10% FBS after 8 hours and incubated for an additional 16 hours at 37 °C. FA-NP loaded with SCR rather than luciferase siRNA was tested as a control to confirm RNA interference. The siRNA concentration of each well was 50 nM. Subsequently, 20 μL cell culture lysis agent was added into each well, followed by allowing cracking for 3 min at room temperature. After the suspension was transferred to a blank 96-well plate, 100 μL luciferase detection agent was added into each well. The suspension was fully mixed for 30 s by vigorous pipetting and was detected using a Tecan Infinite F200 multimode plate reader with biological fluorescence filter at the exposure time of 10 s. The total-protein was extracted and determined according to the BCA Protein Assay Kit.

In vivo fluorescence imaging

Mice bearing subcutaneously implanted Bel-7402 tumors were subjected to fluorescence imaging. Polyplexes (N-NP, P-NP and FA-NP) carrying AF750-labeled SCRwere injected into the tumor-bearing mice*via* tail vein at 400 μg/kg body weight(n = 3). The fluorescence images were captured at different time points on a small animal *in vivo* fluorescence imaging system (Carestream, USA). Before imaging, the mice were anesthetized by intraperitoneal injection of 60 μL 10% chloralic hydras.

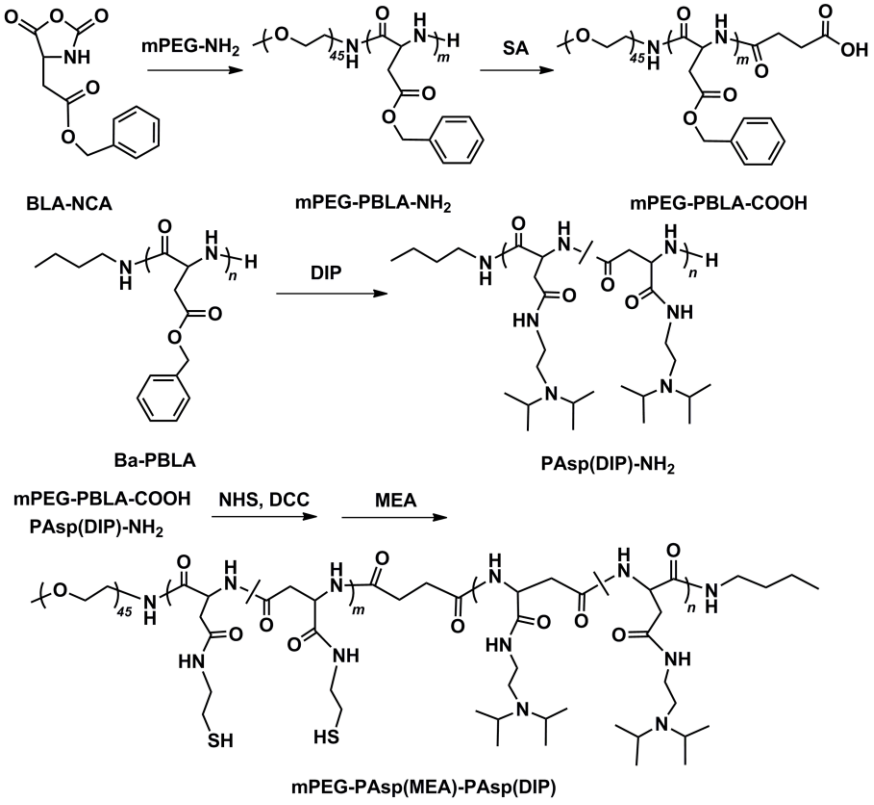
Notes and references

1 W. Wang, D. Cheng, F. Gong, X. Miao, X. Shuai, *Adv.*, *Mater.* 2012, **24**, 115-120.

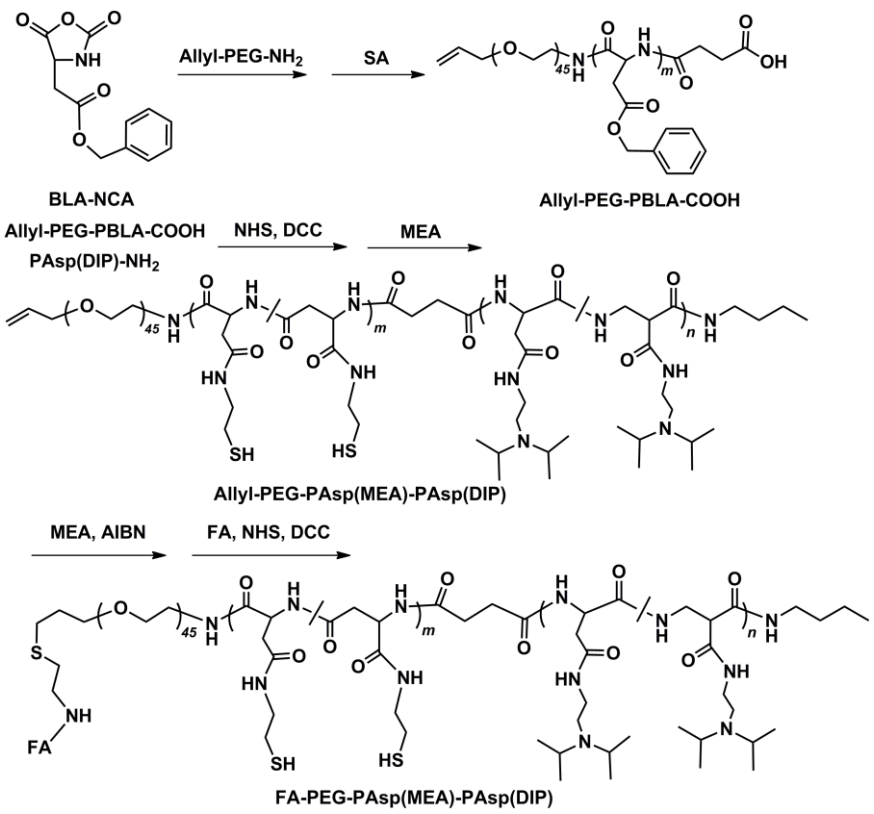
2 Jian Dai, Shudong Lin, Du Cheng, Seyin Zou, and Xintao Shuai, *Angew. Chem. Int. Ed.*, 2011, **50**, 9404-9408.

3 Masataka Nakanishi, Joon-Sik Park, Woo-Dong Jang, Makoto Oba, Kazunori Kataoka, *React. Funct. Polym.*, 2007, **67**, 1361-1372.

4 X. Yang, Y. Chen, R. Y, G. Chen, E. Blanco, J. Gao, X. Shuai, *Polymer*, 2008, **49**, 3477-3485.



Scheme S1 Synthetic route of the non-targeted copolymer mPEG-PAsp(MEA)-PAsp(DIP).



Scheme S2 Synthetic route of the FA-targeted copolymer FA-PEG-PAsp(MEA)-PAsp(DIP).

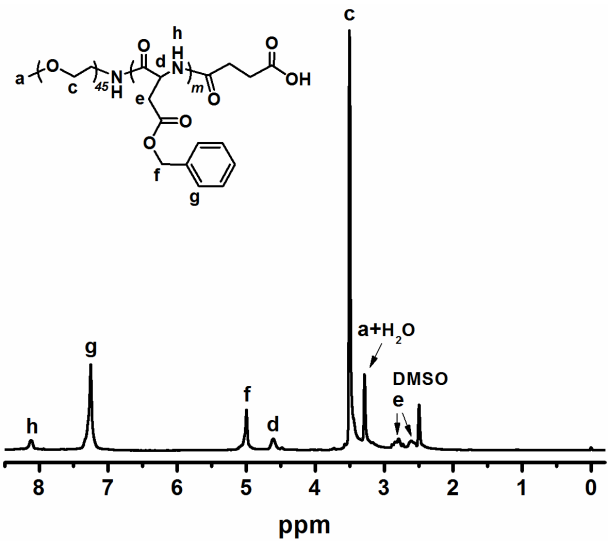


Fig. S1 ^{1}H NMR spectrum of PEG-PBLA-COOH in DMSO-d_6.

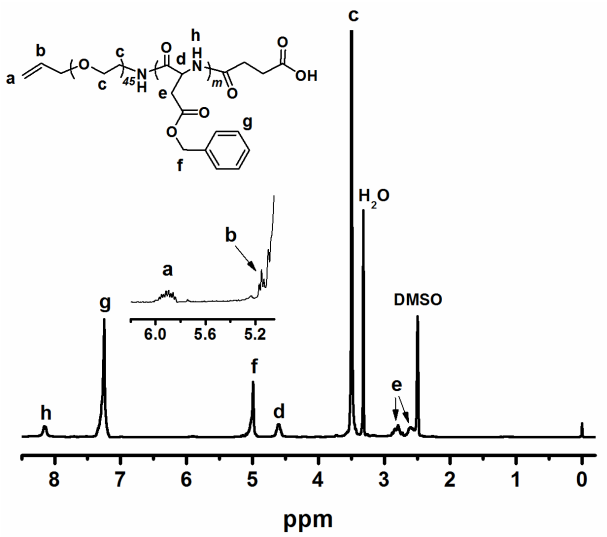


Fig. S2 ^{1}H NMR spectrum of Allyl-PEG-PBLA-COOH in DMSO-d_6.

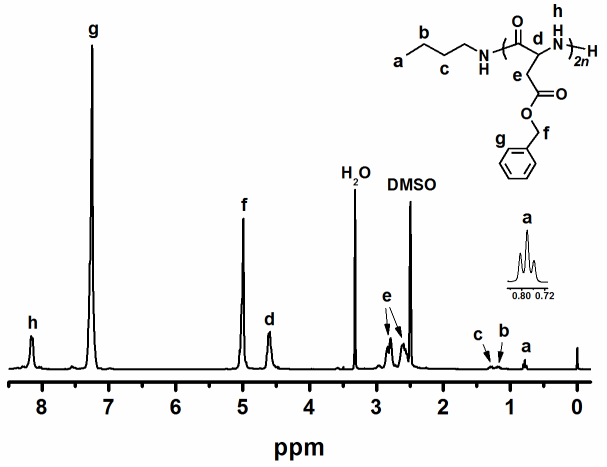


Fig. S3 ^{1}H NMR spectrum of Ba-PBLA in DMSO-d_6.

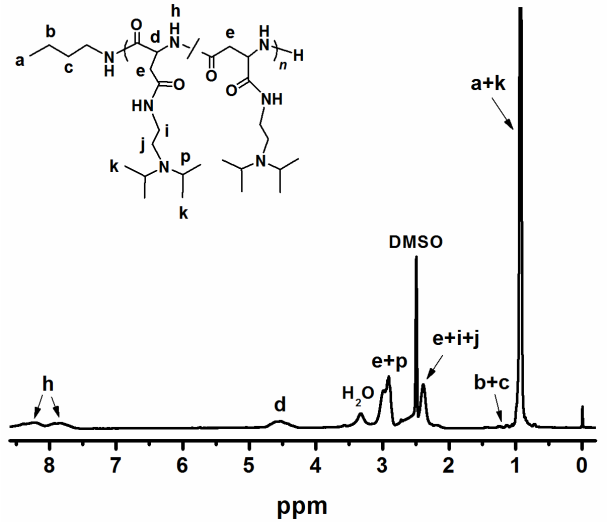


Fig. S4 ^{1}H NMR spectrum of PAsp(DIP) in DMSO-d_6.

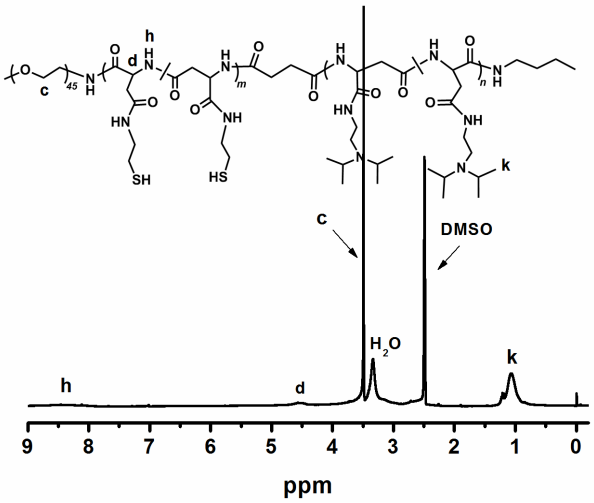


Fig. S5 ^{1}H NMR spectrum of mPEG-PAsp(MEA)-PAsp(DIP) in DMSO-d_6.

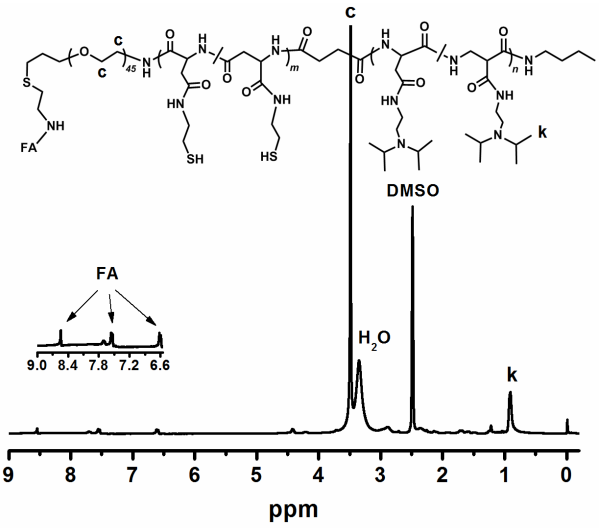


Fig. S6 ^{1}H NMR spectrum of FA-PEG-PAsp(MEA)-PAsp(DIP) in DMSO-d_6.

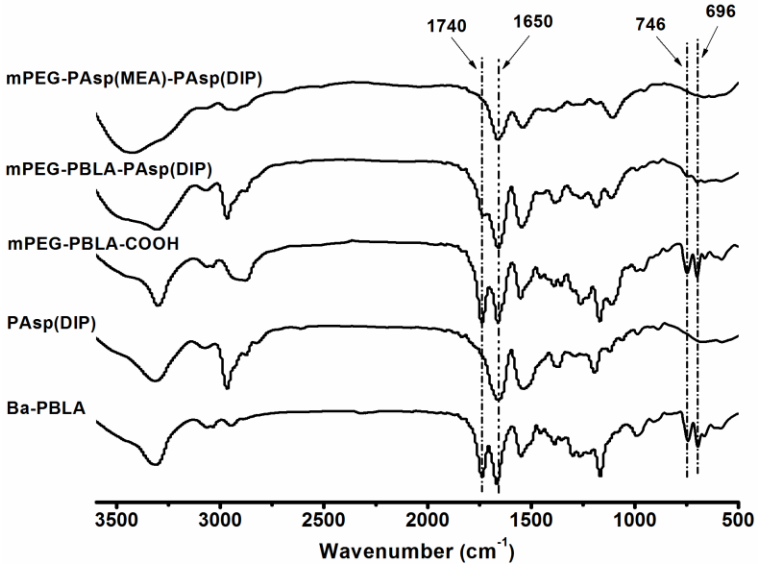
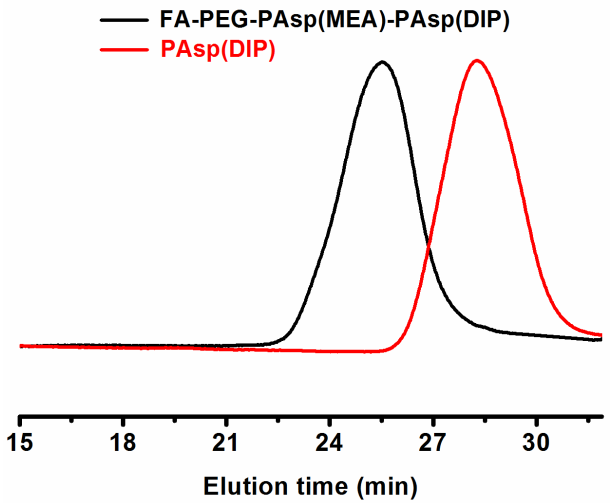


Fig. S7 FTIR spectra of mPEG-PAsp(MEA)-PAsp(DIP) and its prepolymers.



Polymer	M_n[a]	M_n[b]	M_w/M_n[b]
PAsp(DIP)	7100	7900	1.26
FA-PEG-PAsp(MEA)-PAsp(DIP)	10800	12500	1.34

[a]calculated by ^{1}H NMR; [b]calculated by GPC.

Fig. S8 GPC curves of PAsp(DIP) and FA-PEG-PAsp(MEA)-PAsp(DIP) in THF at a flow rate of 1 mL/min.

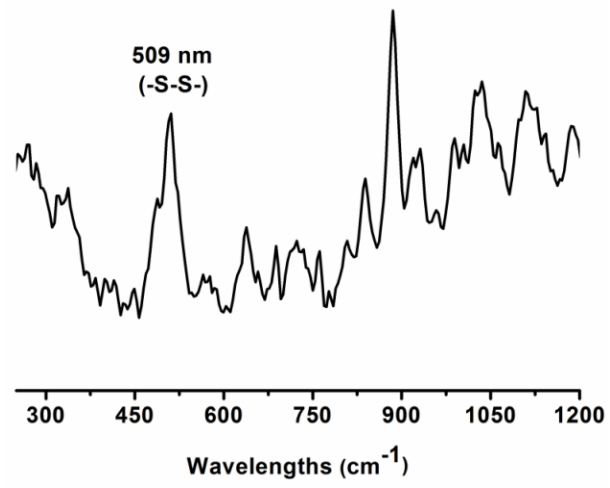


Fig. S9 Raman spectrum of the crosslinked polyplex based on FA-PEG-PAsp(MEA)-PAsp(DIP) showing formation of disulfide bonds.

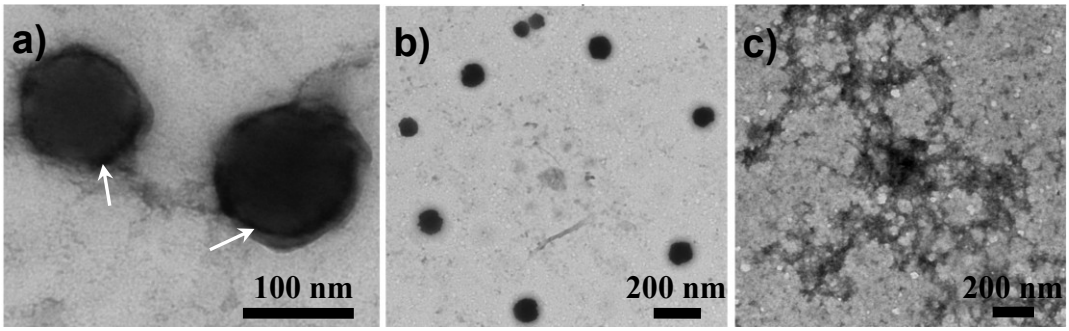


Fig. S10 TEM images of FA-NP (N/P=6) stained with uranyl acetate for 2 min in a) PBS 7.4 + 5 μM GSH; b) PBS 5.0 + 10 mM GSH; c) PBS 7.4 + 10 mM GSH. Incubation time: 2 h. Disulfide linkage structure was identified in a) as a darker circlein interlayer since it was prone touranyl acetate staining [*Water Sci. Technol.*, 2014, **69**, 1775-1787].

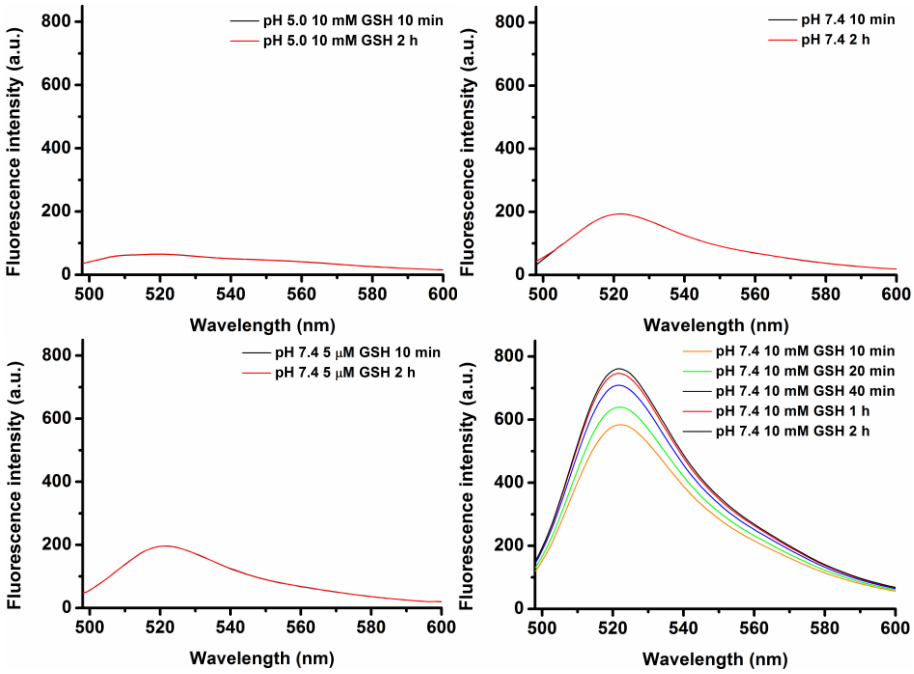


Fig. S11 FITC fluorescence intensity changes of polyplex solutions (FA-NP) at different pH and GSH concentrations. Only in the condition of pH 7.4 +10 mM GSH (cytosol-mimicking environment), the FITC fluorescence intensity increased obviously over time, indicating siRNA release.

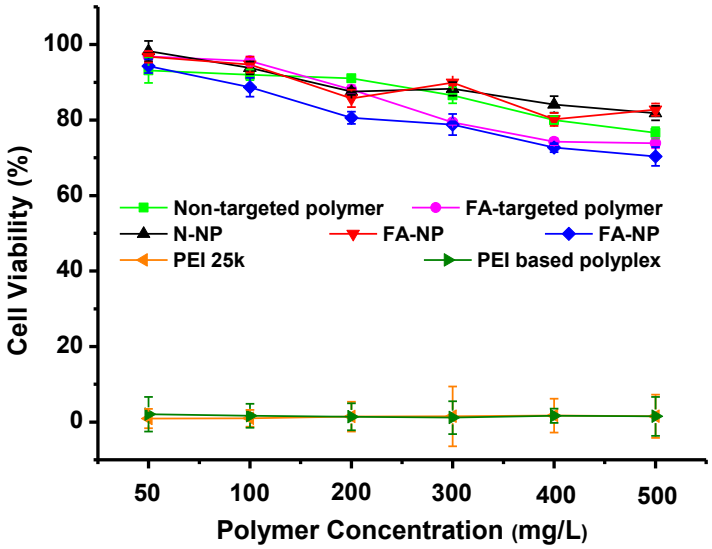


Fig. S12 Cytotoxicity of polymers and polyplexes in Bel-7402 cells at different polymer concentrations. Incubation time: 24 h.

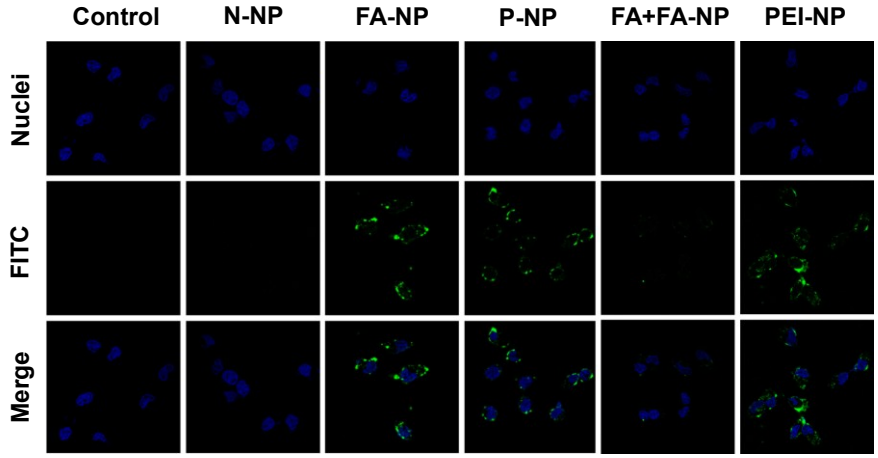


Fig. S13 Laser scanning confocal microscopic images (magnification: 630×) of Bel-7402 cells incubated with different polyplexes at pH 7.4 for 8 h. Blue, nuclei stained with DAPI; green, FITC-SCR. Free FA concentration for FA+FA-NP group: 1 mg/L [*Biomaterials*, 2011, **32**, 2222-2232].

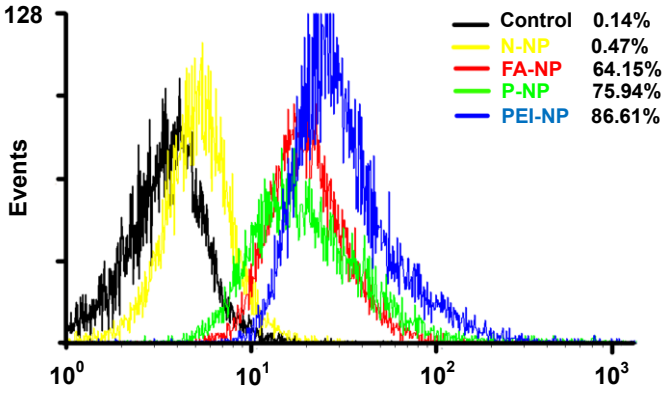


Fig. S14 Quantitative analysis of FITC-positive cells by flow cytometry. Bel-7402 cells were incubated with different polyplexes (N/P=6) for 12 h at the siRNA dose of 25 nM.

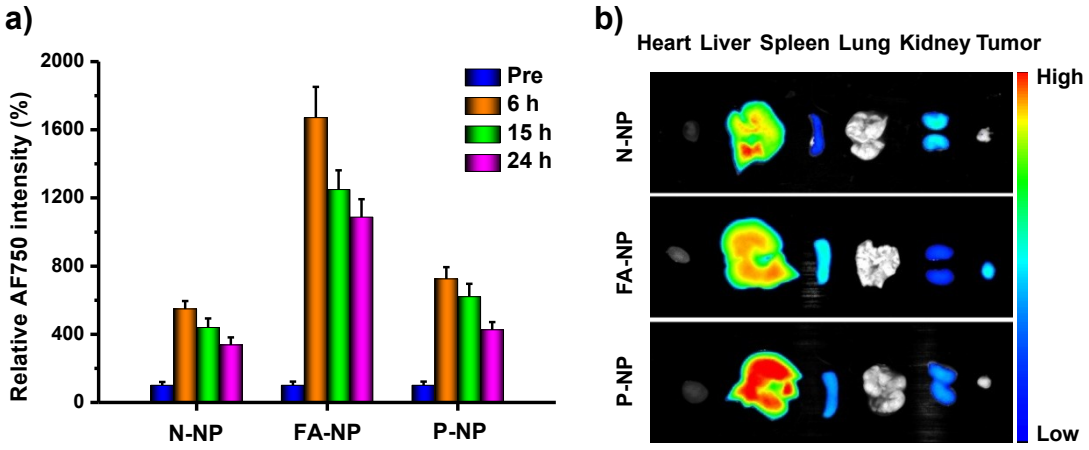


Fig. S15 a) AF750 fluorescence intensities (n = 3) at the tumor sites of nude mice receiving different polyplexes via tail vein injection. siRNA was labeled with AF750. The fluorescence intensities were normalized using the "Pre" group as a standard for each polyplex group. b) *Ex vivo* AF750 fluorescence images of the organs and tumors excised at 24 h post-injection time from the same animal.

Table S1: Averaged particle sizes of FA-NP with or without Interlayer-crosslinking at different pH values. Results are presented as the mean ± SD (n = 5).

Interlayer state	Size/nm at pH 5.0	Size/nm at pH 6.8	Size/nm at pH 7.4
crosslinked	122.4 ± 11.5	128.1 ± 10	138.6 ± 15.2
noncrosslinked	165.6 ± 10.6	366.6 ± 29.9	435.3 ± 32.6