

Supplementary Information for:

## PEG-sheddable polyplex micelles as smart gene carriers based on MMP-cleavable peptide-linked block copolymers

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**Materials.** Dichloromethane, *N*-methyl-2-pyrrolidone (NMP), and diethylenetriamine (DET) were dried over calcium hydride (CaH<sub>2</sub>) and distilled before use. Copper(I) bromide (CuBr, 99%), *N,N,N',N'',N'''*-pentamethyldiethylenetriamine (PMDETA, 98%), and anhydrous *N,N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich and used as received. Fetal bovine serum (FBS), trypsin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO and used as received. The plasmid DNA (pDNA) encoding luciferase with a CAG promoter was amplified in competent DH5 $\alpha$  *Escherichia coli* and purified with a QIAGEN HiSpeed Plasmid MaxiKit (Germantown, MD). Cell culture lysis buffer, luciferase Assay System Kit, and enhanced BCA Protein Assay Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). For cellular uptake and intracellular distribution assay, pDNA was labeled with Cy5 using a Label IT Nucleic Acid Labeling Kit from Mirus Bio Corporation (Madison, WI) according to the manufacturer's protocol. Recombinant human MMP-2 (purity >95%, the specific activity > 1000 pmoles/min/ $\mu$ g) was purchased from Sino Biological Inc. (Beijing, China). *p*-Aminophenyl mercuric acid was purchased from GENMED SCIENTIFICS INC. USA.  $\alpha$ -Methoxy- $\omega$ -azido-poly(ethylene glycol) (PEG<sub>227</sub>-N<sub>3</sub>,

$M_n = 10000$ ,  $M_w/M_n = 1.05$ ) was obtained from JenKem Technology Co., Ltd. (Beijing, China).  $\beta$ -Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) was obtained from Chengdu Enlai Biological Technology Co., Ltd. (Chengdu, China). Peptide G(propargylglycine)-PLGVRG (*alkynyl*-GPLGVRG, Purity 95.36% from HPLC, ESI-MS: calcd. for  $(C_{31}H_{51}N_{10}O_8 + H)^+$ : 692.82; found: 692.7.) was obtained from ChinaPeptides Co., Ltd. (Shanghai, China). All other commercially available solvents and reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. and used as received.

### Sample preparation

*Synthesis of PEG-GPLGVRG-NH<sub>2</sub>*. The peptide *alkynyl*-GPLGVRG (50 mg, 0.072 mmol), PEG<sub>227</sub>-N<sub>3</sub> (0.6 g, 0.06 mmol), PMDETA (31 mg, 0.18 mmol), and anhydrous DMF (3 mL) were charged into a 5-ml Schlenk flask. The mixture was degassed by a freeze-pump-thaw cycle and backfilled with N<sub>2</sub>. CuBr (26 mg, 0.18 mmol) was introduced as a solid under the protection of N<sub>2</sub>. The reaction system was degassed by three freeze-pump-thaw cycles again and sealed under vacuum. Then, the Schlenk flask was placed in a preheated oil bath at 40 °C. After 24 hr, the resulting mixture was precipitated into diethyl ether. After filtration and drying in a vacuum oven overnight at room temperature, the powder was dissolved in water and dialyzed against distilled water for three days using a dialysis bag (MWCO: 6000 Da). The solution was lyophilized, affording PEG-GPLGVRG-NH<sub>2</sub> as a white powder (0.48 g, yield: 74.8%;  $M_{n, GPC} = 12.3$  kDa,  $M_w/M_n = 1.07$ ).

*Synthesis of PEG-GPLGVRG-PBLA*. Block copolymer, PEG-GPLGVRG-PBLA, was prepared via the ring-opening polymerization of BLA-NCA initiated by the terminal amino groups of PEG-GPLGVRG-NH<sub>2</sub>. Firstly, PEG-GPLGVRG-NH<sub>2</sub> initiator was dissolved in a small amount of CH<sub>2</sub>Cl<sub>2</sub>, followed by addition of an excess amount of benzene, and lyophilized to obtain the anhydrous white powder. Then, lyophilized PEG-GPLGVRG-NH<sub>2</sub> (0.107 g, 0.01 mmol) was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (4 mL) followed by addition of BLA-NCA (0.187 g, 0.75 mmol) in DMF (1 mL), and the reaction mixture was stirred for 96 h at 25 °C under argon atmosphere. The resulting polymer was precipitated into diethyl ether. The above dissolution–precipitation

cycle was repeated twice. The final product was dried in a vacuum oven, yielding a white solid (0.21 g, yield: 87.4%;  $M_{n, GPC} = 24.7$  kDa,  $M_w/M_n = 1.15$ ). The actual DP of PBLA segment was determined to be 65 by  $^1\text{H}$  NMR analysis in  $\text{DMSO-}d_6$  (Fig. S2b). Thus, the polymer was denoted as PEG<sub>227</sub>-GPLGVRG-PBLA<sub>65</sub>.

*Synthesis of PEG-GPLGVRG-PAsp(DET).* PEG<sub>227</sub>-GPLGVRG-PBLA<sub>65</sub> (0.1 g, 0.27 mmol of benzyl ester) was dissolved in a small amount of  $\text{CH}_2\text{Cl}_2$ , followed by addition of an excess amount of benzene, and lyophilized to obtain the anhydrous white powder. Then, lyophilized PEG<sub>227</sub>-GPLGVRG-PBLA<sub>65</sub> was dissolved in anhydrous NMP (5 mL), followed by addition of DET (1.46 mL, 50 equiv to benzyl group of PBLA segment). After stirring for 1 h at  $4^\circ\text{C}$ , the reaction mixture was slowly added dropwise into 20% aqueous acetic acid solution (20 mL). The neutralized solution was dialyzed against 0.01M hydrochloric acid solution and subsequently, distilled water at  $4^\circ\text{C}$ . The white powder, PEG-GPLGVRG-PAsp(DET) was obtained after lyophilization (0.1 g, yield: 85.3%;  $M_{n, GPC} = 22$  kDa,  $M_w/M_n = 1.09$ ; eluent: 10 mM Acetic acid + 500 mM NaCl). The actual DP of PAsp(DET) segment was determined to be 64 by  $^1\text{H}$  NMR analysis in  $\text{D}_2\text{O}$  (Fig. S2c). Thus, the polymer was denoted as PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub>.

**Characterization.** All  $^1\text{H}$  NMR spectra were recorded on a Bruker AV300 NMR 300 MHz spectrometer using  $\text{CDCl}_3$ ,  $\text{DMSO-}d_6$ , or  $\text{D}_2\text{O}$  as the solvent. The molecular weight (MW) and molecular weight distribution of PEG-GPLGVRG-PBLA block copolymer were determined by gel permeation chromatography (GPC) equipped with a Waters 1515 pump and a Waters 2414 differential refractiveindex detector. The eluent was DMF with 1 g/L LiBr at a flow rate of 1.0 mL/min. The MW and MW distribution of PEG-GPLGVRG-PAsp(DET) block copolymer were determined by GPC equipped with an Agilent 1260 pump and an Agilent G1362A differential refractiveindex detector. The eluent was ultrapure water with 500 mM NaCl and 10 mM acetic acid at a flow rate of 1.0 mL/min. A series of low polydispersity PEG standards were employed for calibration. Fourier transform infrared (FT-IR) spectra were recorded on a Bruker VECTOR-22 IR spectrometer. The spectra were collected over 64 scans with a spectral resolution of  $4\text{ cm}^{-1}$ .

**Enzymatic cleavage of PEG-GPLGVRG-PAsp(DET).** The cleavage of the block copolymer PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> was performed according to the manufacturer's Activity Assay protocol. Briefly, PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> (2.8 mg, 0.1 μmol) was dissolved in 0.5 mL TCNB buffer (50 mM Tris with 10 mM CaCl<sub>2</sub>, 150 mM NaCl, and 0.05% Brij 35; pH 7.5), followed by addition of MMP-2 (0.5 mL, 2 μg/mL in TCNB buffer) which was activated by APMA before using. The degradation of the block copolymer was investigated by aqueous GPC measurements after incubation for a predetermined time.

**Preparation of PEG-GPLGVRG-PAsp(DET) block copolymer/pDNA polyplex micelles.**

PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> block copolymer and pDNA were separately dissolved in 10 mM HEPES buffer (pH 7.4) as stock solutions. The block copolymer solution was added into pDNA solution for complexation at varying N/P ratios (residual molar ratio of amino groups in block copolymer to phosphate groups in pDNA), followed by incubation overnight at 4 °C. The final pDNA concentrations in all the polyplex micelles were adjusted to 33.3 μg/mL.

**Particle size measurements.** The time-dependent particles sizes of the polyplex micelles were evaluated in the presence of MMP-2 by the dynamic light scattering technique (DLS) using a Zetasizer Nano ZS instrument, equipped with a He-Ne ion laser ( $\lambda = 632$  nm) at a scattering angle of 90°. The PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> polyplex micelles were prepared in HEPES buffer (pH 7.4) at N/P = 2, followed by addition of activated MMP-2 in TCNB. The final concentrations of pDNA and MMP-2 were adjusted to 33.3 μg/mL and 1 μg/mL, respectively. After incubation for predetermined times at 37 °C, the DLS measurements were also conducted at 37 °C.

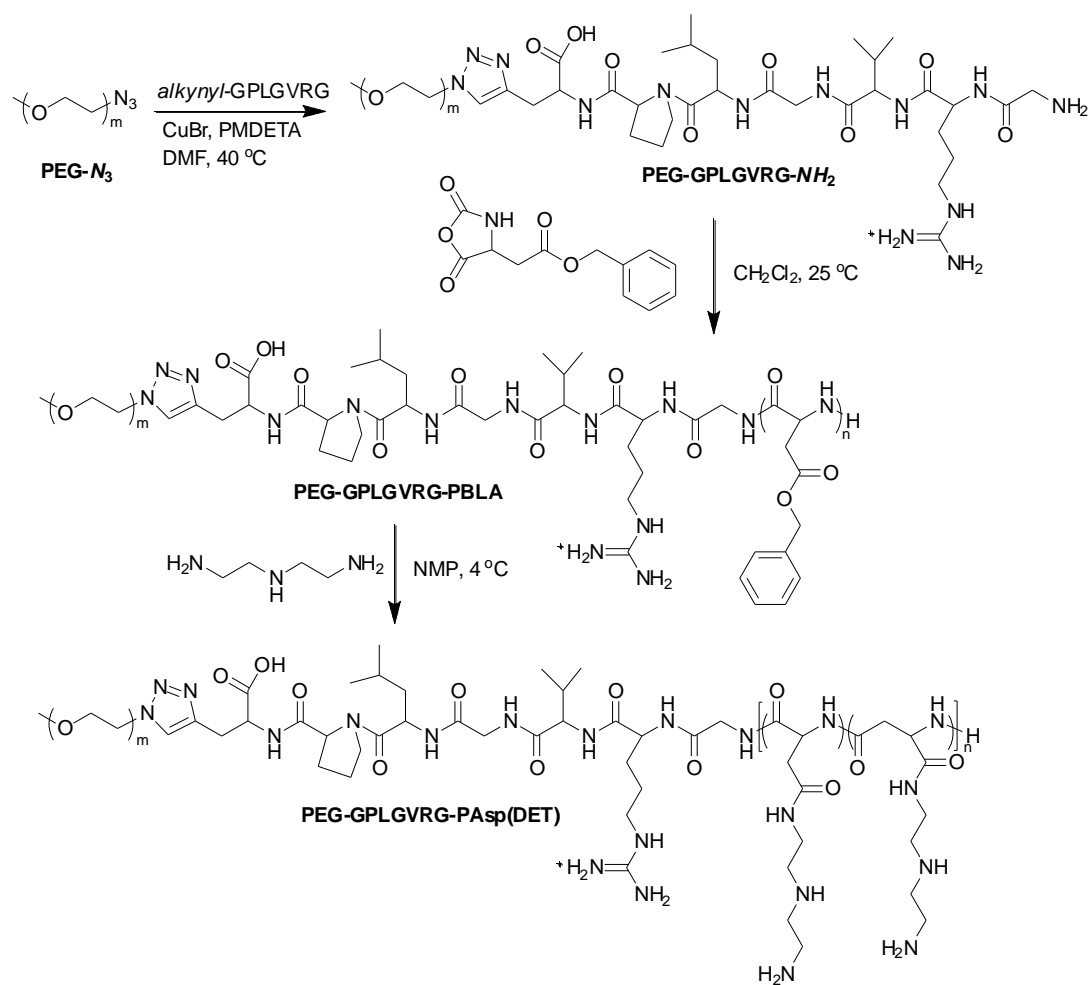
**Cellular uptake.** HeLa cells were seeded in 6-well culture plates at a density of  $1 \times 10^5$  cells/well and incubated overnight in 2 mL DMEM containing 10% FBS in humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The medium was replaced with fresh medium, followed by addition of 150 μL PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> block copolymer/Cy5-labelled pDNA polyplex micelles (33.3 μg pDNA/mL) at varying N/P ratios. Then, activated MMP-2 was added

at the final concentration of 1  $\mu\text{g}/\text{mL}$ . For comparison, cellular uptake studies of PEG<sub>227</sub>-GPLGVRG-PA<sub>sp</sub>(DET)<sub>64</sub> block copolymer/Cy5-labelled pDNA polyplex micelles without MMP-2 were also carried out under the same conditions. After 6 h incubation, the cells were washed 3 times with PBS to remove extracellular Cy5 fluorescence. After detachment by trypsin from the culture plate, the cells were harvested and resuspended in PBS for flow cytometry measurements. Data were analyzed with Flowjo software.

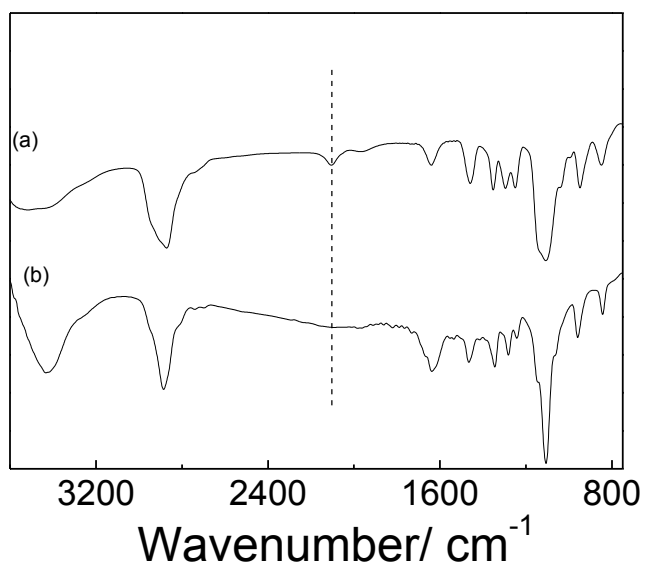
**Intracellular distribution.** Intracellular distribution of polyplex micelles was observed by confocal microscopy. HeLa cells were seeded into a 35-mm glass-bottom culture dish (NEST, China) at a density of  $5 \times 10^4$  cells/well and incubated overnight in 2 mL DMEM containing 10% FBS in humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The medium was replaced with fresh medium, followed by addition of 75  $\mu\text{L}$  PEG<sub>227</sub>-GPLGVRG-PA<sub>sp</sub>(DET)<sub>64</sub> block copolymer/Cy5-labeled pDNA polyplex micelles (33.3  $\mu\text{g}$  pDNA/mL) at N/P = 8 into each cell culture dishes. Then, activated MMP-2 was added at the final concentration of 1  $\mu\text{g}/\text{mL}$ . After 6 h incubation, the medium was removed and the cells were rinsed three times with PBS. Cell nuclei and lysosome were counterstained with DAPI (Blue, Biyotime, Nantong, China) and Lyso-Tracker (Green, Molecular Probes, Eugene, OR), respectively. Then, cells were washed three times with cold PBS and observed with laser scanning confocal laser scanning microscopy (Leica TCS SP5, Germany). For comparison, the observation of intracellular distribution of PEG<sub>227</sub>-GPLGVRG-PA<sub>sp</sub>(DET)<sub>64</sub> block copolymer /Cy5-labeled pDNA polyplex micelles without MMP-2 were also carried out under the same conditions.

**In vitro transfection efficiency.** HeLa cells were seeded on 24-well culture plates ( $2 \times 10^4$  cells/well) and incubated overnight in 400  $\mu\text{L}$  of DMEM containing 10% FBS in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The medium was replaced with 400  $\mu\text{L}$  of fresh medium, followed by addition of 30  $\mu\text{L}$  PEG<sub>227</sub>-GPLGVRG-PA<sub>sp</sub>(DET)<sub>64</sub> block copolymer/pDNA polyplex micelles (33.3  $\mu\text{g}$  pDNA/mL) at various N/P ratios. Then, activated MMP-2 was added at the final concentration of 1  $\mu\text{g}/\text{mL}$ . After 6 h incubation, the medium was exchanged with 400  $\mu\text{L}$  fresh DMEM, followed by another 48 h incubation. The cells were washed with 400  $\mu\text{L}$  of PBS, and lysed in 200  $\mu\text{L}$  of the cell culture lysis buffer. The lysates were centrifuged at 14,000

g for 2 min at 4 °C. Then, 100  $\mu$ L of supernatant was transferred to a 96-well microplate without introducing bubbles into the well. Bioluminescence signals were measured using a 96-well microplate luminometer with an exposure time of 10 s. The amount of total cellular protein was determined by a BCA Protein Assay (Beyotime, Nantong, China). The final results were reported in terms of relative light units (RLU)/mg protein. For comparison, the transfection efficiency of PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> polyplex micelles loading Cy5-labeled pDNA without MMP-2 were also measured under the same conditions.

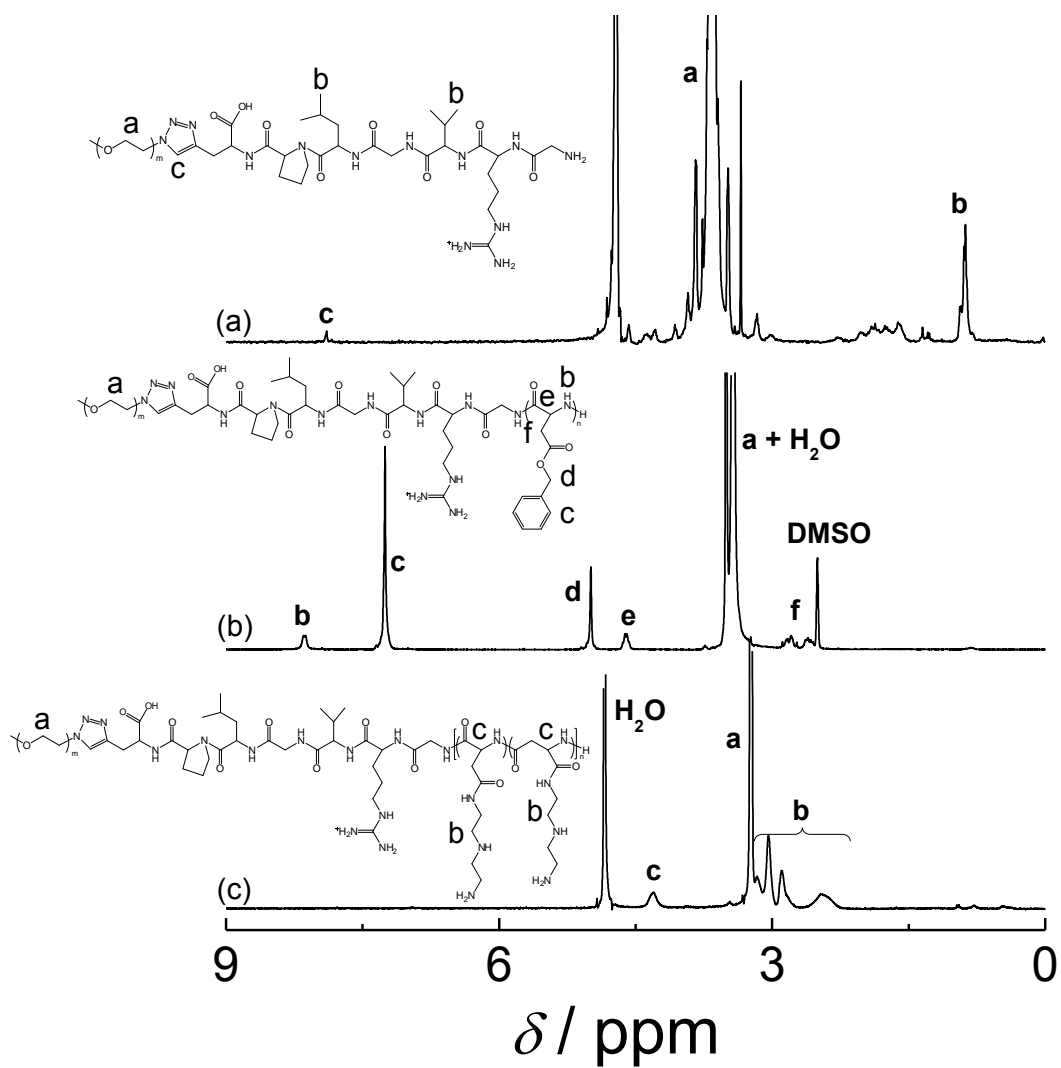


**Scheme S1** Synthetic routes of the MMP-2 cleavable peptide linked block copolymers via click reaction and ring-opening polymerization (ROP).

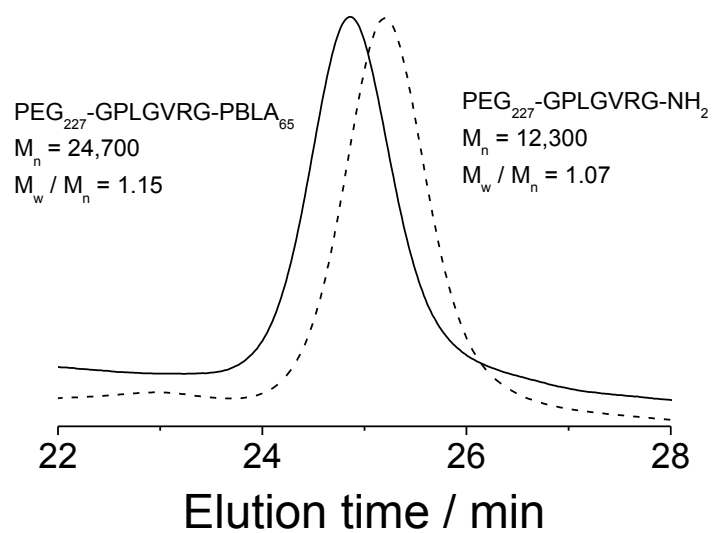


**Fig. S1** FT-IR spectra recorded for (a) PEG-N<sub>3</sub> and (b) PEG-GPLGVRG-NH<sub>2</sub>.

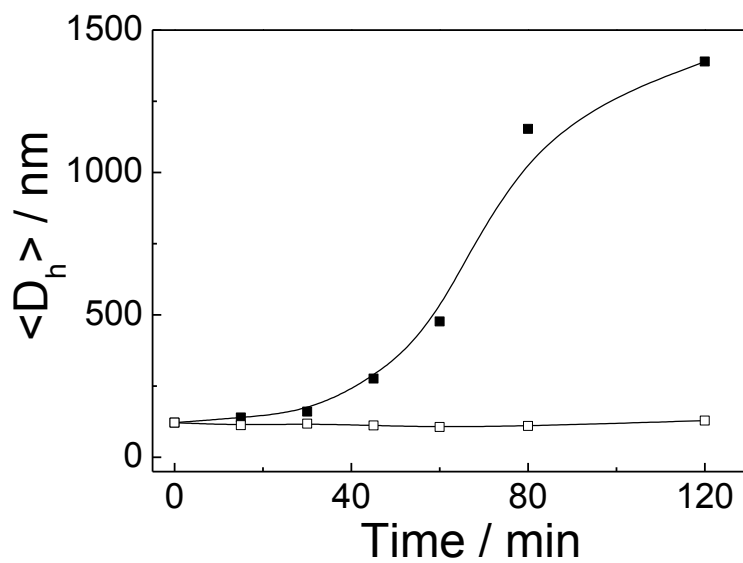




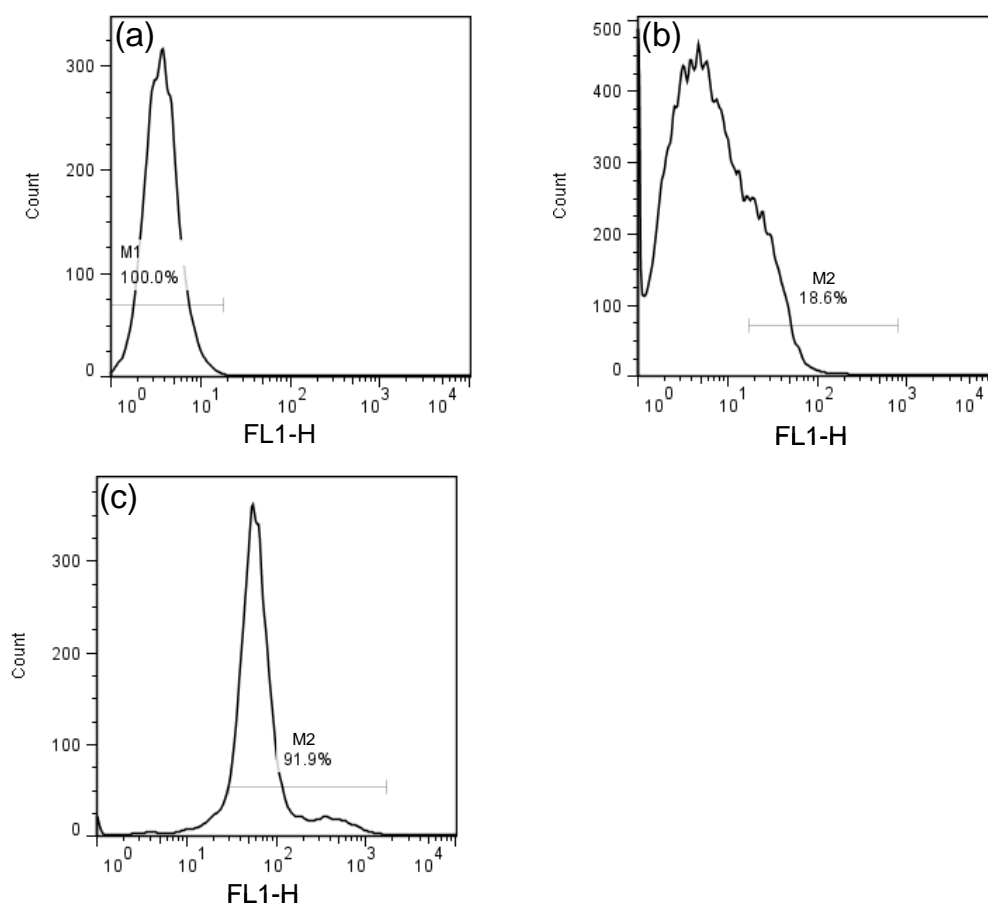
**Fig. S2**  $^1\text{H}$  NMR spectrum recorded for (a) PEG-GPLGVRG- $\text{NH}_2$ , (b) PEG-GPLGVRG-PBLA, and (c) PEG-GPLGVRG-PA<sub>sp</sub>(DET).



**Fig. S3** DMF GPC traces obtained for initiator PEG<sub>227</sub>-GPLGVRG-NH<sub>2</sub> ( $M_n = 12300$ ,  $M_w/M_n = 1.07$ ) and block copolymer PEG<sub>227</sub>-GPLGVRG-PBLA<sub>65</sub> ( $M_n = 24700$ ,  $M_w/M_n = 1.15$ ).



**Fig. S4** Time-dependent intensity-average hydrodynamic diameter,  $\langle D_h \rangle$ , of the PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> block copolymer polyplex micelles at N/P = 2 in the presence of 1  $\mu\text{g/mL}$  MMP-2 (■) and absence of MMP-2 (□) at 37 °C.



**Fig. S5** Typical cellular uptake of PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> polyplex micelles loading Cy5-labelled pDNA at N/P = 8 examined by flow cytometry against HeLa cells (a) without adding micelles as control; (b) PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> polyplex micelles loading Cy5-labelled pDNA in the absence of MMP-2; and (c) PEG<sub>227</sub>-GPLGVRG-PAsp(DET)<sub>64</sub> polyplex micelles loading Cy5-labelled pDNA in the presence of 1  $\mu$ g/mL MMP-2.