

# pH-Sensitive Multi-PEGylated Block Copolymer as a Bioresponsive pDNA Delivery Vector

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## ABSTRACT

**Purpose** A reversibly-PEGylated diblock copolymer, poly(aspartate-hydrazide-poly(ethylene glycol))-block-poly(aspartate-diaminoethane) (p[Asp(Hyd-PEG)]-*b*-p[Asp(DET)]) was reported here for enhanced gene transfection and colloidal stability. The diblock copolymer possessed a unique architecture based on a poly(aspartamide) backbone. The first block, p[Asp(Hyd)], was used for multi-PEG conjugations, and the second block, p[Asp(DET)], was used for DNA condensation and endosomal escape.

**Methods** p[Asp(Hyd-PEG)]-*b*-p[Asp(DET)] was synthesized and characterized by <sup>1</sup>H-NMR. Polyplexes were formed by mixing the synthesized polymers and pDNA. The polyplex size, ζ-potential, and *in vitro* transfection efficiency were determined by dynamic light scattering, ζ-potential measure-

ments, and luciferase assays, respectively. pH-dependent release of PEG from the polymer was monitored by cationic-exchange chromatography.

**Results** The polyplexes were 70–90 nm in size, and the surface charge was effectively shielded by a PEG layer. The transfection efficiency of the reversibly PEGylated polyplexes was confirmed to be comparable to that of the non-PEGylated counterparts and 1,000 times higher than that of the irreversibly PEGylated polyplexes. PEG release was demonstrated to be pH-sensitive. Fifty percent of the PEG was released within 30 min at pH 5, while the polymer incubated at pH 7.4 could still maintain 50% of PEG after 8 h.

**Conclusion** The reversibly PEGylated polyplexes were shown to maintain polyplex stability without compromising transfection efficiency.

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## INTRODUCTION

Gene therapy is a promising approach for treating various diseases, including cancer and hereditary diseases caused by genetic defects. The success of gene therapy depends on the development of gene delivery vectors which can efficiently, selectively, and safely transport therapeutic genes to the target sites. The most efficient gene delivery vectors are viruses, and they are the major subjects being investigated in clinical trials (1). However, viral-based vectors still possess intrinsic safety issues which hamper their practical use in humans (2–5). Non-viral vectors, including polymer-based vectors and liposome-based vehicles, have attracted attention of many researchers in recent years (6,7) due to their lower risks of immunoge-

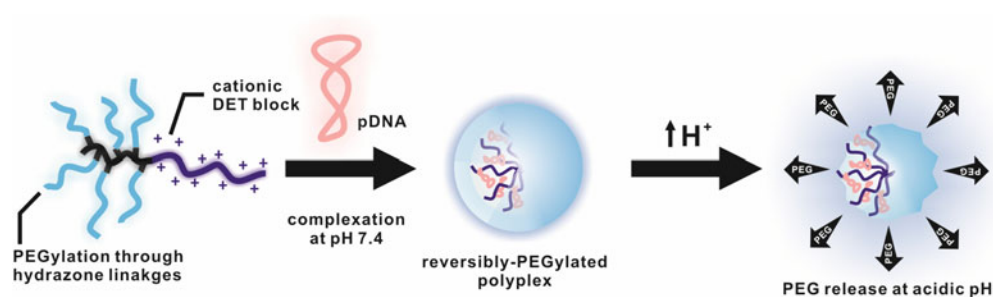
nicity and insertional mutagenesis, higher capability of delivering large-sized DNA molecules, and higher adaptability to chemical production and modification compared to the viral vectors (8,9). Although non-viral vectors have many advantages over virus-based vehicles, the transfection efficiency is still much lower than viruses (10). The low ability to deliver genetic materials can be due to several reasons, for example, low extracellular stability, insufficient endosomal escape, low cellular uptake, and impotency in nuclear entry of therapeutic genes (11). Stability of the complexes forming between non-viral vectors and plasmid DNA (pDNA) can be one of the most crucial factors for successful *in vivo* gene delivery as extracellular stability, which could be related to blood protein interactions, governance of the circulation time of the complexes (12), capability of the complexes to withstand the harsh physiological environments, and, thus, ability for the complexes to reach the therapeutic targets.

In order to increase the colloidal stability of the complexes under physiological conditions, PEGylation is a very common and effective strategy employed by many researchers (13–15). PEGylation has been used extensively on polymer- (16), liposome- (17), or even virus-based (18) delivery vehicles. By forming a dense hydrophilic and sterically hindered PEG palisade around the complex core, the complexes would be able to minimize the interactions with other complexes and the biomolecules in human body (19), and also to endure the high salt concentration in physiological environment (15). On one hand, PEGylation can increase the stability of gene delivery complexes and is crucial for *in vivo* applications, but on the other hand, the transfection efficiency of the PEGylated complexes is always shown to be lower than that of the non-PEGylated counterparts (17,20,21). The compromised transfection efficiency caused by PEGylation is known as PEG dilemma.

Temporary PEGylation of complexes through environmentally sensitive chemical linkages, for example, hydrazone (22), disulfide (23), and ortho ester linkages (24), have been tested by different groups and shown to enhance transfection efficiency. To overcome the PEG dilemma, we have designed and synthesized a reversibly PEGylated diblock copolymer p[Asp(Hyd-PEG)]-*b*-p[Asp(DET)] with a unique

architecture based on a poly(aspartamide) backbone (Fig. 1). The cationic polymer possessing one block, poly(aspartamide) derivative-bearing hydrazides (p[Asp(Hyd)]), for multi-PEG conjugation, and another block, poly(aspartamide) derivative-bearing diaminoethane side chains (p[Asp(DET)]), for DNA condensation and endosomal escape, was used as a bioresponsive vehicle to enhance transfection efficiency and colloidal stability. The hydrazone linkage employed in PEGylation in this study is pH-sensitive and is stable at neutral pH (25), so PEGylation using hydrazone bonds can provide high stability for the polyionic complexes (polyplexes) formed between pDNA and cationic polymers during systemic circulation, and, thus, prolonged circulation time can be achieved. On the other hand, hydrazone linkage is hydrolyzed rapidly at acidic pH, and nanoparticles with size smaller than 200 nm are usually shown to employ the clathrin-mediated endocytosis for cell internalization (26–28); therefore, the PEG chains are expected to dissociate from the polyplexes promptly after endocytosis of the polyplexes as the pH gradually decreases from 7.4 (physiological pH) to 6 (endosomal pH) and then to 5 (lysosomal pH) along the endocytic pathway (29,30). Through the release of PEG from the first block of the polymer at acidic pH, the cationic segment (second block) of the polymer could be more exposed to the negatively charged endosomal membrane. This interaction could trigger the disruption of the endosomal membrane and enhance the endosomal escape of the polyplexes. Furthermore, the reduction of steric hindrance after PEG release could promote efficient release of DNA from the polyplexes following endosomal escape (31). Poly(aspartamide) backbone carrying diaminoethane side chains (p[Asp(DET)]) chosen as the second block for DNA condensation in this study was due to the promising results in terms of *in vitro* and *in vivo* transfection efficiency with very low cytotoxicity shown by recent extensive investigations (21,23,32,33). All these should contribute to a larger amount of DNA localizing at the cytoplasm or even nucleus, and, thus, higher transfection efficiency is expected. By manipulating the decrease in pH along the endocytic pathway and the temporary attachment of PEG through hydrazone linkages, it is expected that the reversibly PEGylated DET-based polycation synthesized

**Fig. 1** Schematic illustration of polyplex formation and PEG release from reversibly PEGylated polyplex in acidic environment.



here could achieve appreciable polyplex stability without compromising transfection efficiency.

## MATERIALS AND METHODS

### Materials

Poly(ethylene glycol) propionaldehyde (Mw 5 kDa) was obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan).  $\beta$ -Benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) was synthesized from L-aspartic acid  $\beta$ -benzyl ester by the Fuchs–Farthing method using triphosgene (34). L-Aspartic acid  $\beta$ -benzyl ester, triphosgene, *n*-butylamine, diethylenetriamine (DET), dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), *N*-methyl-2-pyrrolidone (NMP), trifluoroacetic acid (TFA), benzene, *tert*-butyl carbazate, 2-hydroxypyridine (2-HP), sodium cyanoborohydride (NaBH<sub>3</sub>CN), and branched polyethylenimine (bPEI) (Mw 25 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). *n*-Butylamine, and DET were distilled by conventional methods before use. DCM, DMF, DMSO, and NMP were purchased as anhydrous grade. Dialysis tubings (MWCO's 1,000 and 6,000–8,000) were purchased from Spectra/Por (Rancho Dominguez, CA). Amicon ultra-15 centrifugal filter units (MWCO 30,000) were purchased from Millipore (Billerica, MA). The pDNA coding for luciferase in pGL3-C vector with a SV40 promoter (Promega, Madison, WI) was amplified in competent DH5 $\alpha$  *E. coli* cells and purified using PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA). Luciferase Assay System Kit, CellTiter-Blue® Cell Viability Assay, CytoTox-ONE™ Homogeneous Membrane Integrity Assay, and ethidium bromide (EtBr) were purchased from Promega (Madison, WI). *RC DC* Protein Assay Kit was purchased from Bio-Rad (Hercules, CA). RESOURCE S 1 mL cationic exchange column and AKTA FPLC system were purchased from GE Healthcare.

### <sup>1</sup>H NMR and Gel Permeation Chromatography (GPC) Analyses

The <sup>1</sup>H NMR spectrum of each polymer was obtained with Varian Unity-Inova 400 MHz NMR spectrometer (Palo Alto, CA) with temperature regulated at 25°C or 80°C. Chemical shifts were reported in ppm relative to the residual protonated solvent resonance. Polymer molecular weight distributions were monitored using TOSOH TSK-gel G3000PWXL and G4000PWXL columns with an internal refractive index detector. DMF with 10 mM LiCl was used as the eluent at a flow rate of 1 ml/min at 40°C. PEG standards were used for calibration.

## Synthesis of Polymers

### *p*[Asp(Hyd-PEG)]-*b*-*p*[Asp(DET)]

Poly( $\beta$ -benzyl-L-aspartate) (PBLA) was synthesized by ring-opening polymerization using  $\beta$ -benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) and *n*-butylamine as the monomer and initiator, respectively. To aim at polymerization degree (DP) of about 30, appropriate amount of BLA-NCA was dissolved in DMSO in a round-bottom flask under argon atmosphere at a concentration of 50 mg/mL. *n*-butylamine was dissolved in DMSO in another flask under argon. The dissolved BLA-NCA solution was then quickly added to the flask containing *n*-butylamine using cannula in a stream of argon, and the mixture was stirred at 40°C for 24 h for polymerization. The product mixture was slowly added into cold diethylether. The precipitated polymer was then subjected to centrifugation. The precipitation procedure was repeated for at least 5 times to purify the polymer. The white crude solid obtained was dissolved in benzene and then freeze-dried. The degree of polymerization was determined to be 27 from the peak intensity ratio of the aryl protons of the benzyl groups (C<sub>6</sub>H<sub>5</sub>–,  $\delta$ =7.2–7.3 ppm) to the methyl group protons of *n*-butylamine (CH<sub>3</sub>–,  $\delta$ =0.8 ppm) in <sup>1</sup>H NMR spectrum taken in DMSO-d<sub>6</sub> at 25°C (data not shown).

The side chains of PBLA were then substituted with Boc-protected hydrazide through an aminolysis reaction. PBLA, 10 equiv of *tert*-butyl carbazate and 5 equiv of 2-hydroxypyridine with respect to the benzyl group of PBLA were dissolved in DMF under argon and stirred at 45°C for 48 h. The polymer was precipitated in cold diethylether and then dissolved in methanol and dialyzed against methanol using MWCO 1,000 dialysis tubing overnight to remove the 2-hydroxypyridine. The purified polymer was then freeze-dried in a mixture of methanol and benzene. The degree of substitution was determined to be 92.6% (25/27) from the peak intensity ratio of the *tert*-butyl protons of the Boc protecting groups ((CH<sub>3</sub>)<sub>3</sub>C–,  $\delta$ =1.2 ppm) to the methyl group protons of *n*-butylamine (CH<sub>3</sub>–,  $\delta$ =0.8 ppm) in <sup>1</sup>H NMR spectrum taken in DMSO-d<sub>6</sub> at 25°C (data not shown).

The *p*[Asp(Hyd-Boc)] polymer was subsequently used for the second polymerization. The polymer was dissolved in DMF under argon, and appropriate amount of BLA-NCA (aiming at DP=100) dissolved in a mixture of DMF (<10% total volume) and DCM was added in a stream of argon. The reaction mixture was stirred at 35°C for 4 days. The degree of polymerization was determined to be 106 from the peak intensity ratio of the aryl protons of the benzyl groups (C<sub>6</sub>H<sub>5</sub>–,  $\delta$ =7.2–7.3 ppm) to the methyl group protons of *n*-butylamine (CH<sub>3</sub>–,  $\delta$ =0.8 ppm) in <sup>1</sup>H NMR spectrum taken in DMSO-d<sub>6</sub> at 25°C (data not shown). To protect the *N*-terminal

amino group of the polymer, the polymer was dissolved in NMP, and acetic anhydride (100 equiv) was added under argon. The reaction mixture was stirred at 40°C for 2 h.

To deprotect the Boc group, ice-cold TFA was added to the polymer and stirred for 30 min at 0°C. TFA was then removed under vacuum, and the polymer was dissolved in DCM, precipitated in diethylether, and freeze-dried in benzene. The resulting polymer had a polydispersity of 1.27 as determined by GPC (data not shown). To conjugate PEG to the polymer through the acid-sensitive hydrazone linkage, 10 equiv of PEG propionaldehyde and the polymer were mixed in DMSO and stirred for 24 h. The unconjugated PEG was then removed by precipitating the polymer mixture in methanol. The number of PEG conjugated to each polymer was confirmed to be 6 from the peak intensity ratio of the aryl protons of the benzyl groups ( $C_6H_5-$ ,  $\delta=7.2-7.3$  ppm) to the methylene protons of PEG ( $-OCH_2CH_2-$ ,  $\delta=3.5$  ppm) in  $^1H$  NMR spectrum taken in DMSO- $d_6$  at 25°C (data not shown).

The side chains of the second PBLA block were then substituted with DET through aminolysis reaction in NMP for 30 min at 15°C. The polymer was then precipitated in diethylether and subsequently dried and dissolved in 10 mM PBS (pH 7.4) and dialyzed against PBS. The buffer salts and free PEG were then removed by Amicon ultra-15 centrifugal filter units (MWCO 30,000), and the polymer as a hydrochloride salt form was then freeze-dried in distilled water. The substitution was confirmed by the disappearance of the aryl protons of the benzyl groups ( $C_6H_5-$ ,  $\delta=7.2-7.3$  ppm) and the emergence of methylene protons of the DET moieties ( $-CH_2CH_2NHCH_2CH_2-$ ,  $\delta=2.6-3.6$  ppm) in the  $^1H$  NMR spectrum taken in  $D_2O$  at 80°C (data not shown).

#### $p[Asp(PEG)]-b-p[Asp(DET)]$

The irreversibly PEGylated polycation was synthesized in the similar way described in the previous section. The reversible hydrazone linkage between PEG and polymer was reduced by  $NaBH_3CN$  in 10 mM PBS (pH 7.4), purified using centrifugal filter units and then freeze-dried.

#### Preparation of Polyionic Complex (Polyplex)

The stock solutions were prepared by dissolving pDNA and synthesized polymers in 10 mM Tris-HCl buffer (pH 7.4) at concentrations of 50  $\mu g/mL$  and 10  $mg/mL$ , respectively. Polyplexes were formed by adding different amounts of polymer solutions (1/3 total volume) to pDNA solutions (2/3 total volume) at various N/P ratios and incubated at ambient temperature for at least 30 min before experiments. The final concentration of pDNA in all the samples was kept

at 33  $\mu g/mL$ . The polyplexes were formed in the same way in all experiments unless stated otherwise.

#### Gel Retardation and EtBr Exclusion Assay

Polyplexes with different N/P ratios were loaded on a 0.8 wt% agarose gel containing 0.004% ethidium bromide and electrophoresed at 100 V for 45 min with a running buffer of 40 mM Tris-acetic buffer with 2 mM EDTA. The migrated pDNA was visualized by UV. In the EtBr dye exclusion assay, polyplex samples formed at 33  $\mu g$  pDNA/mL with different N/P ratios were adjusted to 2.3  $\mu g$  pDNA/mL with 2.5  $\mu g$  EtBr/mL in 10 mM Tris-HCl buffer (pH 7.4) and incubated at ambient temperature overnight in dark. The fluorescence intensity of the samples excited at 510 nm was measured at 590 nm using a spectrofluorometer (599B, Perkin-Elmer, MA). The reported relative fluorescence intensity ( $F_r$ ) was calculated by

$$F_r = (F_{\text{sample}} - F_0)/(F_{100} - F_0),$$

where  $F_{\text{sample}}$  is the fluorescence intensity of the polyplex samples,  $F_{100}$  is the free pDNA without any polymer, and  $F_0$  is the background without pDNA and polymer.

#### Dynamic Light Scattering (DLS) and $\zeta$ -potential Measurements

The DLS and  $\zeta$ -potential were measured using a Zetasizer Nano-ZS (Malvern Instruments, UK) at a detection angle of 173° at ambient temperature, and a He-Ne ion laser ( $\lambda=633$  nm) for the incident beam. Polyplex samples formed at 33  $\mu g$  pDNA/mL with different N/P ratios were adjusted to 14  $\mu g$  pDNA/mL using 10 mM Tris-HCl buffer (pH 7.4). The polyplex samples (750  $\mu L$ ) were transferred into low volume cuvettes and then followed by the measurement. The data obtained were analyzed by the cumulant method, and the hydrodynamic diameters of samples were then calculated by the Stokes-Einstein equation, and the sizes were reported as Z-average diameters. To measure the  $\zeta$ -potential, the sample solutions were transferred to a Dip cell (Malvern Instruments, UK), and the electrophoretic mobility was measured; the  $\zeta$ -potential was then calculated by the Smoluchowski equation:

$$\zeta = 4\pi\eta v/\varepsilon,$$

where  $\eta$  is the viscosity of the dispersant,  $v$  is the measured electrophoretic mobility, and  $\varepsilon$  is the dielectric constant of the dispersant.

#### Polyplex Stability in Physiological Salt Conditions

Polyplex samples were formed using different polymers at 33  $\mu g$  pDNA/mL with the same procedure as described in

the previous section, except distilled water was used as the medium. After 30 min complexation at room temperature, the samples were diluted to 10  $\mu\text{g}$  pDNA/mL using distilled water. 10X Tris-HCl buffer saline (100 mM, 1.5 M NaCl, pH 7.4) was added to mimic the physiological salt concentration. The polyplex sizes were monitored using DLS for 12 h at 25°C, and the results were reported as Z-average diameters.

### Time- and pH-Dependent PEG Release Study

Reversibly PEGylated polymer was incubated at 10 mM Hepes buffer (pH 7.4) or sodium acetate buffer (pH 5) at 37°C for 24 h. Aliquots were withdrawn at specific time-points and diluted with ice-cold Hepes buffer (pH 7.4). Samples were then immediately subjected to purification by RESOURCE S 1 mL cationic exchange column, and the unbound fractions containing free PEG were saved for subsequent analysis (35). Briefly, 41.6  $\mu\text{L}$  of 1 N barium chloride was added to 187.5  $\mu\text{L}$  of the unbound fraction; 20.8  $\mu\text{L}$  of 0.1 N iodide solution was subsequently added and mixed. The samples were allowed to sit at room temperature for 15 min, and then the absorbance was measured at 535 nm using plate reader (SpectraMax M2<sup>e</sup>, Molecular Devices, CA). 100% PEG release was calculated based on sample incubated with 0.1 M HCl at 37°C overnight.

### pH-Sensitive Release of PEG and Polyplex $\zeta$ -Potential

Polyplex samples were formed using different polymers at 33  $\mu\text{g}$  pDNA/mL with the same procedure described using distilled water as the medium. After 30 min complexation at room temperature, the samples were diluted to 10  $\mu\text{g}$  pDNA/mL using distilled water. 10X Tris-acetate buffer (100 mM, pH 7.4) or 10X sodium acetate buffer (100 mM, pH 5) was added to adjust the samples to appropriate pH. These two buffers were chosen to keep the buffer anions consistent. The change of polyplex charges was monitored for 4 h at room temperature.

### Serum Nuclease Resistance Assay

The polyplexes were formed with the same procedure described above using distilled water. pH was adjusted to 7.4 with 10X Tris-HCl buffer (100 mM, pH 7.4), and 10% (v/v) of FBS was added; then the samples were incubated at 37°C. At specific time-points, naked DNA and polyplexes were withdrawn, and 1  $\mu\text{L}$  of 5 M EDTA was added to stop the nuclease activity; then the samples were kept frozen at -20°C until gel electrophoresis. 20 units of heparin were used to release pDNA from the polyplexes and incubated for 30 min at room temperature prior to electrophoresis.

### In Vitro Transfection

MDA-MB-231 human breast cancer cell line was obtained from ATCC and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were seeded at 30,000 cells/well on 24-well and incubated for 24 h. After the cells reached to about 60% confluence, polyplexes were prepared with different polymers synthesized at varying N/P ratios and incubated at room temperature for at least 30 min before experiment. Polyplex solution (30  $\mu\text{L}$ ) was added to the cells after medium substitution with 270  $\mu\text{L}$  of fresh DMEM containing 10% FBS. The pDNA amount for each well was kept at 1  $\mu\text{g}$ . The polyplexes were allowed to incubate with the cells for 6 h. The polyplex-containing medium was then removed, and 1 mL of fresh DMEM with 10% FBS was added to each well. The cells were then maintained at 37°C for another 42 h before luciferase assay. For luciferase assay, the cells were washed with 1x PBS (1 mL) two times after medium removal. 140  $\mu\text{L}$  of lysis reagent was then added to each well, and the cells were incubated at room temperature for 15 min. 100  $\mu\text{L}$  of luciferase substrate was added to 20  $\mu\text{L}$  of the lysate, and the luminescence intensity was measured by an Orion microplate luminometer (Berthold Detection Systems, Oak Ridge, TN). The obtained luciferase activity was then normalized with the amount of total proteins present in the lysates determined by the RC DC Protein Assay Kit. The degree of polymerization of the second block of PEG-*b*-p[Asp(DET)] used in this experiment was determined to be 80 by <sup>1</sup>H NMR.

### Metabolic Cytotoxicity

MDA-MB-231 cells were seeded at 3,000 cells/well on a 96-well plate in DMEM containing 10% FBS and incubated for 24 h. Polyplex solutions (10  $\mu\text{L}$ ) prepared at N/P ratios were added to the cells after fresh medium substitution (90  $\mu\text{L}$ ). The cells were incubated with polyplexes for 6 h before refreshing medium. The cells were incubated for another 18 h, and the assay was carried out according to the manufacturer's protocol (CellTiter-Blue® Cell Viability Assay). The fluorescence signals were measured 3 h after adding the dye. The results were represented as percentages of metabolic rate determined using untreated cells.

### LDH Release Study

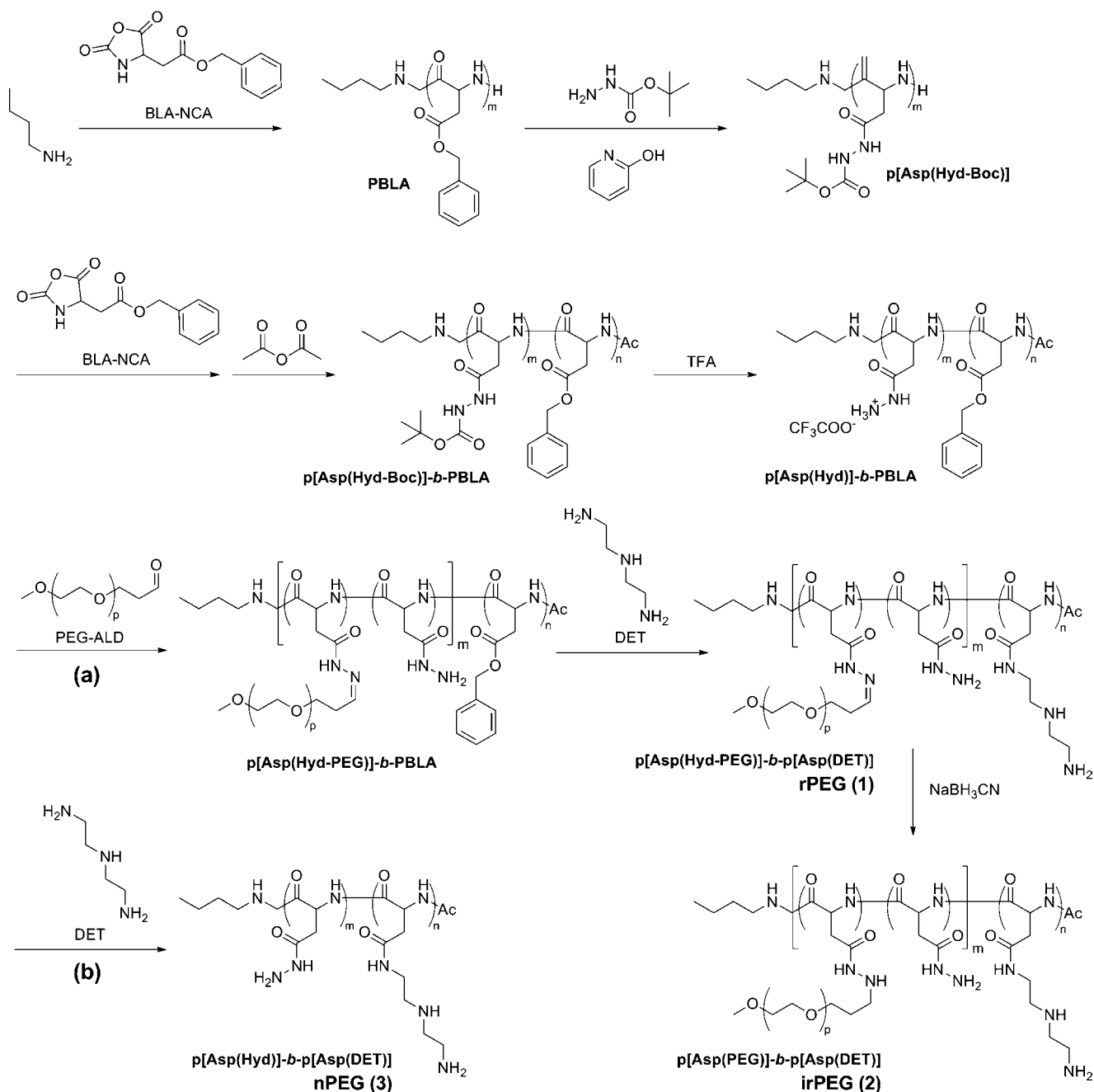
MDA-MB-231 cells were seeded at 3,000 cells/well on 96-well plates in DMEM containing 10% FBS and incubated for 24 h. After 24 h, medium was removed, and cells were washed with 100  $\mu\text{L}$  1x PBS. Serum-free DMEM (90  $\mu\text{L}$ ) was added, and polymer solutions (10  $\mu\text{L}$ ) prepared at

different amine concentrations were subsequently added to the cells. The cells were incubated with the polymer solutions for 6 h. The assay was then carried out according to the manufacturer's protocol (CytoTox-ONE™ Homogeneous Membrane Integrity Assay). Maximum LDH release was determined from cells treated with lysis solution supplied from the assay kit. Results were reported as percentages of maximum LDH release.

## RESULTS

### Synthesis of p[Asp(Hyd-PEG)]-*b*-p[Asp(DET)] (Scheme 1)

The synthesis of PBLA was carried out using BLA-NCA and *n*-butylamine as the monomer and initiator, respectively. The degree of polymerization was confirmed to be



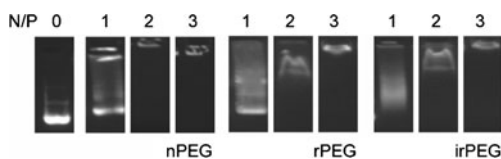
**Scheme 1** (a) Synthetic route of reversibly PEGylated polymer (rPEG (1)), and irreversibly PEGylated polymer (irPEG (2)). (b) Synthetic route of non-PEGylated polymer (nPEG (3)).



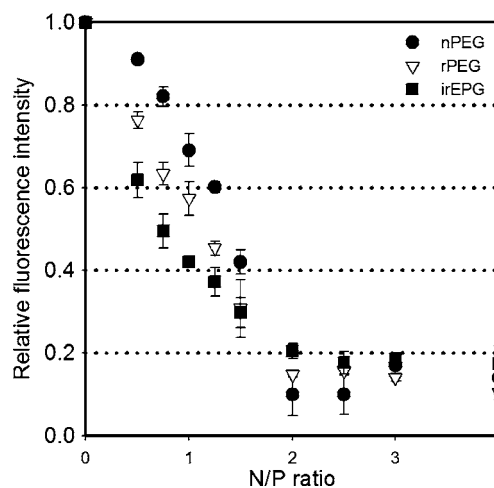
27 by  $^1\text{H}$  NMR. Molecular weight distribution was estimated by GPC, and the distribution was unimodal. To introduce hydrazide moieties to the polymer for subsequent PEGylation, Boc-protected hydrazide was used to prevent undesired initiation at the free hydrazide moieties rather than the terminal amino group at the second polymerization. Since the conversion percentage using Boc-hydrazide alone was not satisfactory, 2-hydroxypyridine (2-HP) was utilized to help raise the conversion degree.  $^1\text{H}$  NMR confirmed that the conversion was 92.6% with the help of 2-HP. The p[Asp(Hyd-Boc)] was then used as a macro-initiator to start the second polymerization of PBLA, and the degree of polymerization was estimated to be 106; GPC revealed a unimodal molecular weight distribution. After Boc group deprotection, conjugation of PEG to the polymer was achieved through hydrazone linkage between the deprotected hydrazide and the aldehyde group from PEG propionaldehyde. After purification, GPC showed that there was no free PEG present, and  $^1\text{H}$  NMR estimated that there were about 6 PEGs conjugated per polymer molecule. The aminolysis of the second block of PBLA was carried out at low temperature ( $15^\circ\text{C}$ ) for very short time (30 min) in order to prevent the release of PEG from the polymer with the presence of excess amine.  $^1\text{H}$  NMR confirmed the aminolysis reaction was successful, and there were 5–6 PEG chains attached on each polymer. The irreversibly PEGylated polymer was synthesized in the same way, and the hydrazone linkages between PEG chains and the polymer backbone were reduced by  $\text{NaBH}_3\text{CN}$  at the final step.

### Formation of Polyplexes

Polyplexes were formed by mixing each polymer solution with pGL3-C pDNA solution directly at different N/P ratios. Agarose gel electrophoresis showed that the bands of free pDNA disappeared at N/P ratio of 2, while the pDNA was completely retarded at N/P ratio of 3 for all different polyplexes, as shown in Fig. 2, which showed that all pDNA was trapped successfully inside the polyplex particles. As the N/P ratio here was calculated solely based on the number of amino groups on the polymer excluding free hydrazide groups and the results are similar to that obtained from homopolymer containing only amino groups from DET side chains (23), we show that hydrazide does not participate in DNA condensation at pH 7.4. These results are consistent with published data (22). EtBr molecules are



**Fig. 2** Agarose gel retardation analysis of the polyplexes.

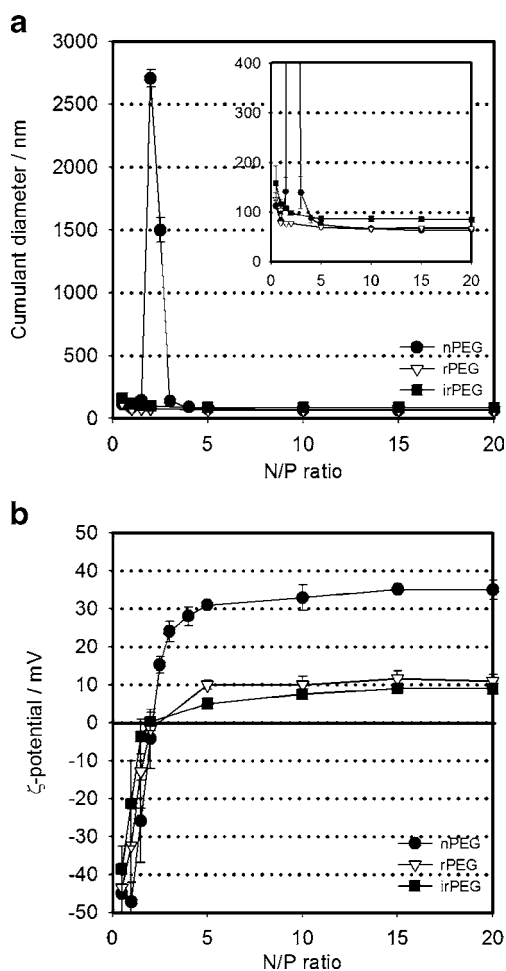


**Fig. 3** Ethidium bromide exclusion assay on the polyplexes prepared from nPEG, rPEG, and irPEG polymers. Relative fluorescence intensity vs N/P ratio. Results are expressed as mean  $\pm$  SEM ( $n=3$ ).

known to emit fluorescence upon interaction with DNA. Also, during pDNA condensation, there is a coil-to-globular structural transition, and the globular structure inhibits the intercalation of DNA by EtBr, resulting in decreased fluorescence emission; therefore, EtBr exclusion assay was employed to determine the condensation degree of pDNA at different N/P ratios quantitatively. The fluorescence data obtained using different polyplexes are shown in Fig. 3. All polyplexes exhibit similar patterns in terms of pDNA condensation ability. The relative fluorescence intensity dropped from N/P ratio of 0 to N/P ratio of 1.5 and reached a plateau at N/P ratio of 2, which indicates that pDNA achieved maximum condensation at N/P ratio of 2, where the ratio of positive charge to negative charge was 1:1, as there was only one protonated amine on each DET side chain at pH 7.4. These results were consistent with the results obtained using gel electrophoresis.

### Size and $\zeta$ -Potential of Polyplexes

Physical characteristics of polyplexes are closely related to their therapeutic performance, and, thus, it is essential to determine the size and surface potential of the polyplexes. DLS measurement data was shown in Fig. 4a. The size of polyplexes gradually decreased to 70–90 nm when N/P ratio was larger than 2 for reversibly PEGylated (rPEG) and irreversibly PEGylated (irPEG) polyplexes. With non-PEGylated (nPEG) polyplexes, N/P ratio of at least 4 was required to bring the size down to the same range. At low N/P ratio range (0.5–3), the sizes of the rPEG and irPEG polyplexes were below 150 nm without aggregation; however, the size of nPEG polyplexes was significantly different from that of the PEGylated polyplexes. The size of the



**Fig. 4** Size and surface potential of the polyplexes prepared at various N/P ratios. **(a)** Cumulant diameters and **(b)**  $\zeta$ -potential. Results are expressed as mean  $\pm$  SEM ( $n=3$ ).

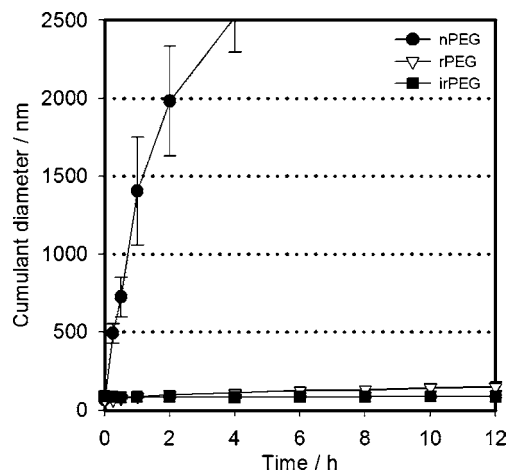
nPEG polyplex went up to about 2,700 nm at N/P ratio of 2, which represented significant aggregation at this N/P ratio. Considering that at pH 7.4, diaminoethane side chain was only mono-protonated, the overall charge of the polyplexes formed at N/P ratio of 2 should be theoretically neutral. This was also confirmed by  $\zeta$ -potential measurements that the surface potential was very close to zero (Fig. 4b). At this critical N/P ratio and without the steric hindrance provided by PEG, the polyplexes experienced the minimal repulsive force among one another, and the counterion screening effect caused the neutral polyplex particles to aggregate.

$\zeta$ -potential measurement data shown in Fig. 4b revealed that the surface potential of the polyplexes increased with N/P ratio. All polyplexes have near neutral surface potential at N/P ratio of 2, and the  $\zeta$ -potential values reached plateau at N/P ratio of 5. The values were maintained the same up to N/P ratio of 20. The significant difference observed in the  $\zeta$ -potential data was the difference between PEGylated and non-PEGylated poly-

plexes. The surface potentials of PEGylated (rPEG and irPEG) polyplexes were kept below +10 mV, which was much lower compared to +30 mV obtained with non-PEGylated (nPEG) polyplexes. This result suggests that the charge on the cationic block of the polymer is efficiently shielded by the PEG moieties.

### Polyplex Stability in Physiological Salt, and Serum Conditions

To investigate the stability of the polyplexes in salt condition, polyplexes were formed in distilled water, and the pH and salt concentrations were changed to 7.4 and 150 mM, respectively, after 30 min complexation time, and the size was monitored using DLS for 12 h. As shown in Fig. 5, the addition of salt instantaneously induced the nPEG polyplexes to aggregate. The size increased from around 70 nm to micron range within 1 h after addition of salt. In the case of rPEG and irPEG polyplexes, the rapid size increase could not be observed, which implies that PEGylation was necessary to keep the polyplexes intact, at least to prevent aggregation, in physiological salt condition. For the PEGylated polyplexes, the size of irPEG polyplexes stayed the same for 12 h after salt addition, while the size of rPEG polyplexes grew from 70 nm to about 150 nm after 12 h. Although the size of rPEG polyplexes increased, it was still much smaller than the size of nPEG polyplexes after salt addition. It was also interesting to observe that even though the size of rPEG polyplexes increased, the PDI values obtained after 12 h were similar or even lower than the initial values, while the PDI values for nPEG polyplexes increased dramatically after aggregation (data not shown). The impact of serum on particle stability was also investigated using DLS, and preliminary data showed that the nPEG polyplexes aggregated significantly, and particle



**Fig. 5** Time-dependent change of size of the polyplexes prepared at N/P ratio of 20 and incubated with 150 mM NaCl Tris-HCl buffer (pH 7.4) at 25°C. Results are expressed as mean  $\pm$  SEM ( $n=3$ ).



size polydispersity became very high after 12 h incubation with 10% FBS. The PEGylated polyplexes, on the other hand, showed higher stability in serum-containing medium. The particle sizes of irPEG polyplexes incubating in serum and serum-free medium are very comparable and stayed below 100 nm after 12 h incubation, while the size of the rPEG polyplexes increased to about 500 nm (data not shown). The increase in size for the rPEG polyplexes in both experiments might be due to the dynamic structure of the polymer, as hydrazone-conjugated PEG is still hydrolysable at pH 7.4.

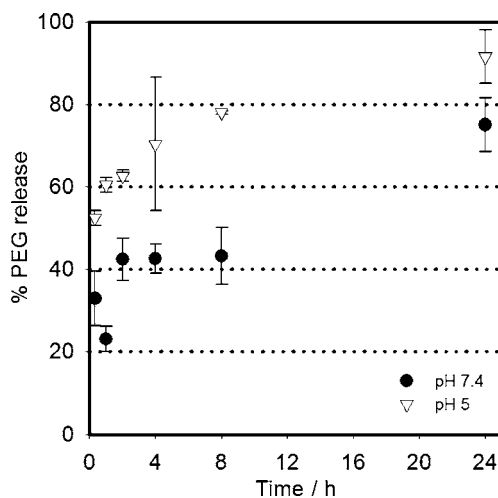
### Time- and pH-Dependent PEG Release Study

As PEGylation of polyplex is required for prolonged systemic circulation and rapid release of PEG inside the cells is preferable for high transfection efficiency, it is necessary to monitor the PEG release from the polymer at different pHs. To quantitatively measure the release of PEG at different pH, the reversibility PEGylated polymer (rPEG) was incubated at 37°C for 24 h at pH 7.4 and pH 5 to mimic the physiological and endosomal pH, respectively. The release profiles at pH 7.4 and pH 5 are shown in Fig. 6. A more rapid release of PEG at the initial stage of the experiment was observed at pH 5 compared to pH 7.4. 50% of total amount of PEG was released within 30 min at pH 5, while the amount of PEG released remained below 50% after 8 h at pH 7.4. Although the number of PEG chains required to stabilize the polyplex during circulation is still unclear, it is true that the reversibly PEGylated polymer here showed the ability to maintain the 50% PEG at pH 7.4 for 8 h, which may be enough for systemic circulation for the polyplexes to reach the targets for therapeutic effects. Moreover, the polymer could release

the PEG shield very rapidly at pH 5, which corresponds to the acidic pH at the endo/lysosomal compartments, and this indicates that the polyplexes might be able to benefit from the intracellular prompt release of PEG to facilitate the endosomal escape of the polyplexes to avoid degradation and also to enhance the release of pDNA from the polyplexes.

### pH-Sensitive Release of PEG and Polyplex $\zeta$ -Potential

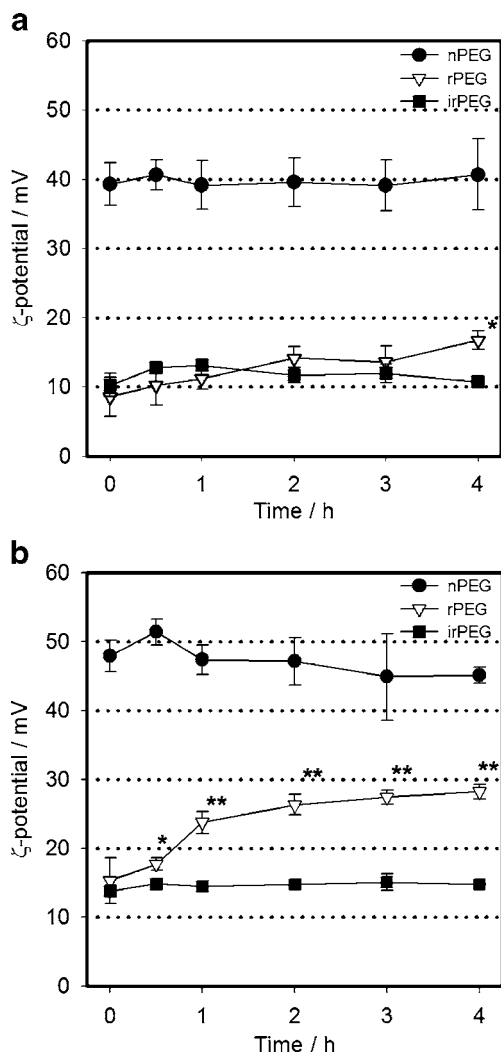
The value of polyplex surface potential is related to the amount of PEG present on the polyplex. Higher PEG density on the polyplex should be able to shield the charge more efficiently, resulting in lower  $\zeta$ -potential. On the other hand, when the PEG chains are detached, the charge of the polyplex will be more exposed to the surroundings, and the  $\zeta$ -potential value measured should then be higher. As hydrazone linkage is hydrolyzed at a faster rate at acidic pH than at neutral pH, monitoring the polyplex surface potential would be useful for confirming PEG conjugated through hydrazone bond is released from the polyplexes more rapidly at low pH. Time-dependent change of surface potentials of different polyplexes is shown in Fig. 7. As buffers affect the  $\zeta$ -potential measurement, it would be more reliable to compare the change of  $\zeta$ -potential of different polyplexes at the same pH. Fig. 7a shows that the amount of PEG released from rPEG polyplexes at pH 7.4 caused significant difference in  $\zeta$ -potential compared to irPEG polyplexes only until  $t=4$  h ( $*P<0.05$ ), but not within the first 3 h. However, Fig. 7b shows that the difference in  $\zeta$ -potential between rPEG and irPEG polyplexes at pH 5 was significant ( $*P<0.05$  and  $**P<0.005$ ) starting at  $t=0.5$  h. These results suggest that the rate of PEG release from the polyplexes at pH 5 was higher than that at pH 7.4, and also the PEG released at pH 5 within the first 4 h could contribute to significant increase in  $\zeta$ -potential.



**Fig. 6** Time- and pH-dependent PEG release from the reversibly PEGylated polymer at pH 5 and pH 7.4 at 37°C. Results are expressed as mean  $\pm$  SEM ( $n=3$ ).

### Serum Nuclease Resistance

Physically, it was confirmed from the DLS,  $\zeta$ -potential measurements and PEG release study that the reversibly PEGylated polyplexes showed satisfactory stability at physiological conditions. Another challenge for polyplex to successfully deliver therapeutic genes to targets is to protect the DNA from degradation by the abundant nuclease present throughout the human body; therefore, the polyplexes were further studied for the ability to resist serum nuclease degradation. Reversibly PEGylated polyplexes were incubated at 37°C with 10 mM Tris-HCl buffer (pH 7.4) containing 10% FBS. Polyplexes were withdrawn at the indicated time-point. The DNA was released from the polyplexes through counter polyanion exchange using heparin as the anionic macromolecules. As shown in

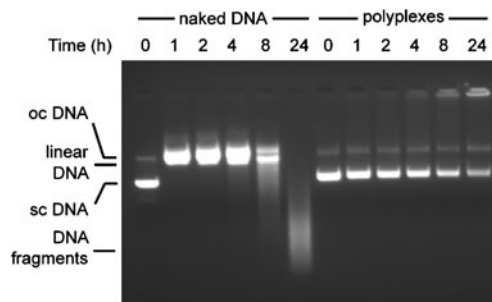


**Fig. 7** Time- and pH-dependent change of  $\zeta$ -potential at (a) pH 7.4 and (b) pH 5 of polyplexes prepared at N/P ratio of 20 at 25°C. Results are expressed as mean  $\pm$  SEM ( $n=3$ ). \* $P<0.05$  and \*\* $P<0.005$ .

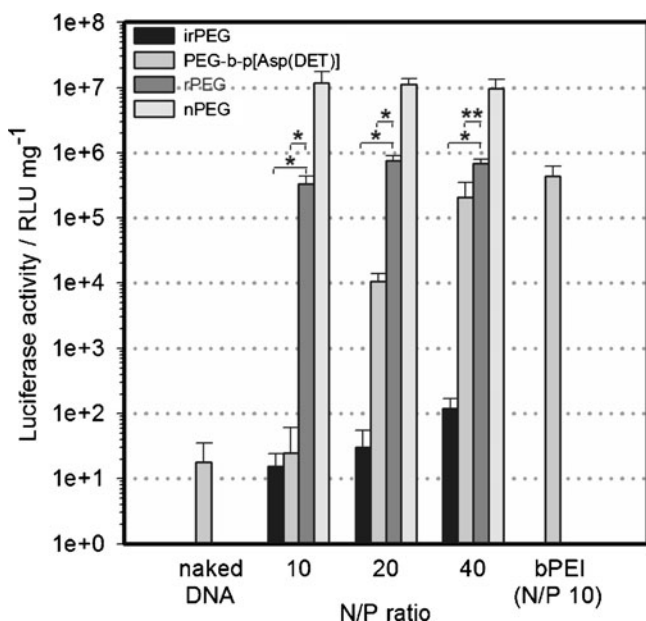
Fig. 8, the supercoiled (sc) form of naked pDNA disappeared completely after 1 h incubation, and all the DNA was changed to the open circular (oc) and linear forms. The band intensity of the oc DNA and linear DNA remained about the same for 4 h and then started to decrease. DNA fragments due to DNA degradation appeared as smear on the agarose gel started to appear at 4 h, and the DNA was completely degraded after 24 h incubation, as only DNA fragments could be observed at this time-point. Compared to naked DNA, the polyplexes showed a very different result. As shown in the gel image, the polyplexes protected the DNA from degradation by serum nuclease for at least 24 h under the experimental conditions. There was no DNA fragment and linear DNA observed at any time-points. Also, there was no significant increase in the intensity of the bands of the oc DNA, which showed that most of the pDNA remained as the intact sc form when complexed with polymer.

## In Vitro Transfection

The transfection efficiency of the polyplexes formed using the polymers synthesized, nPEG, rPEG, and irPEG, was compared to that of the polyplexes formed using PEG-*b*-p [Asp(DET)] and branched polyethylenimine (bPEI) (25 kDa) in MDA-MB-231 breast cancer cell line. bPEI is a control commonly used for comparing transfection efficiency, while PEG-*b*-p[Asp(DET)] is a diblock copolymer that showed promising *in vitro* and *in vivo* gene delivery ability and negligible toxicity (21,33). The polyplexes were formed at N/P ratios of 10, 20, and 40 for this transfection experiment. bPEI polyplexes were formed at N/P ratio of 10 as determined to be the optimal N/P ratio for transfection without causing massive cell death. Fig. 9 shows that the most prominent transfection agent in this study was nPEG polyplex. This agreed with our hypothesis that polyplexes without PEGylation should show the best transfection ability compared to their PEGylated counterparts. The polyplexes formed with irPEG polycations showed the lowest transfection efficiency, which is 5 orders of magnitude lower than that of non-PEGylated polyplexes in this experiment. With the same number of PEG chains, the PEG chains were equipped to rPEG polycations through pH-sensitive hydrazone linkage. The hydrazone hydrolysis and subsequent PEG release were confirmed by the PEG release study to be pH sensitive as discussed in previous sections. With this environmental sensitivity characteristic, it is expected that the polyplexes formed with rPEG polycations should achieve similar results to nPEG-formed polyplexes. As seen in Fig. 9, at various N/P ratios, the transfection efficiency of the rPEG polyplexes was about 4 to 5 orders of magnitude higher than that of irPEG polyplexes (\* $P<0.01$ ) and 1 order of magnitude lower than that of nPEG counterpart. This indicates that polyplexes can achieve a more comparable level of transfection to the non-PEGylated polyplexes through the reversible attachment of PEG chains to the polyplexes even there is a high density of PEG molecules protecting the polyplexes. Moreover, the nPEG and rPEG polyplexes



**Fig. 8** Agarose gel retardation analysis of naked DNA and polyplexes after incubation with 10% FBS. oc DNA and sc DNA represent open circular DNA and supercoiled DNA, respectively.



**Fig. 9** Transfection efficiency analysis of the polyplexes in MDA-MB-231 cell line. pDNA concentration was kept at 1  $\mu\text{g}/\text{well}$  in transfection experiment. bPEI (Mw 25 kDa) was used as a control. Results are expressed as mean  $\pm$  SEM ( $n=3$ ). \*\* $P<0.05$  and \* $P<0.01$ .

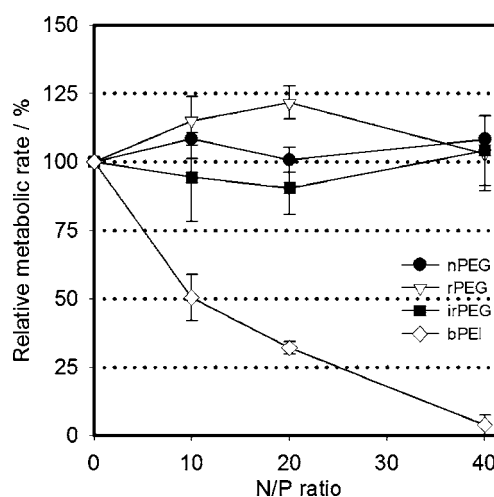
showed higher and similar transfection efficiency compared to bPEI, respectively, while causing minimal toxicity, which will be discussed below. Compared to the thoroughly investigated PEG diblock polymer, PEG-*b-p*[Asp(DET)], rPEG polyplexes have an advantage in terms of transfection efficiency. The difference as shown in Fig. 9 was significant at lower N/P ratios, e.g. 10 and 20 (\* $P<0.01$ ). When N/P ratio increased, however, the transfection ability of the diblock copolymer polyplexes became more comparable to the rPEG polyplexes. Within the N/P ratio range tested, increasing N/P ratio did not affect the transfection efficiency of the nPEG and rPEG polyplex systems very substantially. High transfection level could be achieved as low as N/P ratio of 10 for the nPEG and rPEG polyplexes. On the contrary, N/P ratio of 40 was required for PEG-*b-p*[Asp(DET)] polyplexes to achieve a similar result to rPEG polyplexes.

### In Vitro Cytotoxicity

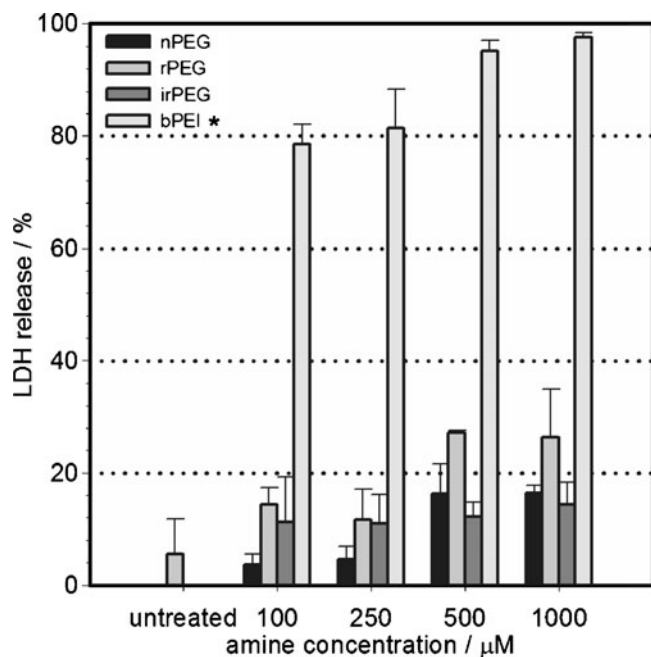
Regarding the cytotoxicity of the polyplexes formed with the polymers synthesized, cytotoxicity assays based on cellular metabolism and LDH release were carried out using MDA-MB-231 cell line. bPEI was used as a control for both assays. The relative metabolic rate of cells treated with different kinds of polyplexes at the same N/P ratio range used in transfection experiment is shown in Fig. 10. Among the polyplexes formed with the DET-based polycations, nPEG, rPEG, and irPEG, no significant difference

in toxicity was observed. Almost 100% cell viability based on metabolism was observed, even at N/P ratio of 40, which was the maximum N/P ratio tested, in MDA-MB-231 cell line. The data revealed that PEGylation of DET-based polycations did not affect the toxicity caused by the polymers, and this might be due to the intrinsic low toxic nature of the DET-based polymers. In contrast, the metabolic toxicity caused by bPEI polyplexes was significantly higher than that caused by DET-based polymer polyplexes at every N/P ratio tested. At the N/P ratio used in transfection experiment, N/P 10, there was already 50% cell death caused by the bPEI polyplexes. The toxicity increased with the amount of bPEI used. Cell viability dropped to 30% at N/P ratio of 20 and less than 10% at N/P ratio of 40.

It is well known that cationic polymers can interact and disrupt cellular membrane. LDH release through the leaky cell membrane damaged by cationic polymers could be used to evaluate the membrane toxicity caused. The membrane toxicity produced by different free polymers at a range of amine concentration is shown in Fig. 11. At amine concentration of 100  $\mu\text{M}$ , bPEI caused about 80% LDH release. The percentage increased to almost 100% when 1000  $\mu\text{M}$  of bPEI was used. DET-based polymers all showed significant lower membrane toxicity (<30%) compared to bPEI (\* $P<0.01$ ), although there was a trend that the LDH release percentage increased with the concentration of amino groups. As a general difference among the different DET-based polymers within the tested concentration range was not present, it could not be concluded that PEGylation could reduce the membrane cytotoxicity of DET-based polymers. The LDH release assay result was consistent with the metabolic toxicity result that DET-based



**Fig. 10** Metabolic toxicity analysis of the polyplexes prepared at different N/P ratios in MDA-MB-231 cell line. pDNA concentration was kept at 0.25  $\mu\text{g}/\text{well}$  in this experiment. bPEI (Mw 25 kDa) was used as a control. Results are expressed as mean  $\pm$  SEM ( $n=3$ ).



**Fig. 11** LDH release caused by polymers at various amino group concentrations in MDA-MB-231 cell line. bPEI (Mw 25 kDa) was used as a control. Results are expressed as mean  $\pm$  SEM ( $n=3$ ). \* $P<0.001$ .

polymers causing minimal and significantly lower toxicity compared to bPEI.

## DISCUSSION

To develop a gene delivery vehicle which is sufficient for *in vivo* application, PEGylation of the vehicle is necessary for increasing systemic circulation time and physiological stability. Although PEGylation can enhance polyplex stability and facilitate the escape from the recognition and clearance of the reticuloendothelial system, it hampers the transfection efficiency of the vehicle. This is probably due to the decreased cellular uptake of the polyplexes and the difficulty in releasing DNA intracellularly as a result of the steric hindrance of PEG molecules. In this regard, a pH-sensitive multi-PEGylated cationic polymer with a unique architecture was synthesized and tested for *in vitro* gene delivery efficiency in this study. The diblock copolymer was composed of two functional segments: hydrazide functionalized block (p[Asp(Hyd)]) for multi-PEGylation through pH-sensitive hydrazone linkages, and p[Asp(DET)] block for DNA condensation and enhanced endosomal escape. PEGylation of the cationic polymer through hydrazone linkages was restricted to one of the two blocks of the polymer, while the second cationic block was kept intact for DNA condensation. This unique synthesis strategy is different from the common ways many investigators employed for multi-PEGylation by conjugating PEG chains

randomly on cationic polymers using reactive amines as the reaction sites (14,20,36,37). The advantages of this restricted PEGylation approach include maintaining the cationic charge density of the polymers after PEG conjugation and minimizing the steric hindrance produced by PEG during DNA condensation (38). These may affect the overall polyplex stability and eventually transfection efficiency. PEG propionaldehyde with molecular weight of 5 kDa was employed in this study as many investigators showed that 5 kDa PEG was sufficient to reduce particle sizes, effective for charge shielding and polyplex stabilization in physiological ionic strength environments compared to lower molecular weight PEG (14). Moreover, molecular weight of 5 kDa was demonstrated to be the minimum PEG molecular weight to avoid plasma protein interaction with nanoparticles (39). The reversibly PEGylated polymer prepared here was expected to provide polyplex stability through PEGylation while releasing the PEG molecules as soon as the polyplexes enter the acidic intracellular compartments, such as endosomes and lysosomes, in order to maintain a high transfection efficiency.

The success of incorporation of DNA into polyplexes using the polymers synthesized was shown in the gel retardation and ethidium bromide exclusion analyses (Figs. 2, 3). The polyplexes obtained from the polymers were about 70–90 nm at N/P ratio equals 5 or greater (Fig. 4a), thereby making them suitable for further *in vivo* applications. Moreover, the  $\zeta$ -potential of the polyplex was efficiently shielded by the high density PEG layer with a reduction from +30 mV to +10 mV as shown in Fig. 4b. Note that the surface charge potential of polyplexes formed from DNA and diblock copolymer, PEG-*b*-p[Asp(DET)], was at about the same value (about +10 mV) (40). The relatively small positive values implied that the PEG shielding was still not 100% complete. The failure to further reduce surface potential by the higher density PEG palisade produced by the multi-PEGylated polymer can be due to the lower molecular weight PEG (5 kDa) employed in this study compared to the 12 kDa PEG used for the synthesis of PEG-*b*-p[Asp(DET)]. Even with higher PEG density, the shorter PEG-formed layer can just provide a comparable charge shielding effect as the longer PEG-formed layer (lower density) does. The small positive surface potential might still produce nonspecific interaction between the polyplexes and negatively charged species inside the body; therefore, PEG with higher molecular weight could be employed for multi-PEGylation in order to further reduce the surface potential, but it may also lower the overall transfection efficiency of the vehicle. The pH-responsive nature of the PEG release from the polymer was confirmed by PEG release study and  $\zeta$ -potential measurements. The polyplexes or free polymers were incubated at pH 7.4 and pH 5 to mimic the physiological pH and acidic



endosomal pH, respectively, in these experiments. At neutral pH, the amount of PEG attached to the polymer could still maintain at more than 50% for about 8 h at 37° C, while the PEG chains were released from the polymer at a much faster rate at acidic pH (50% release within 30 min) (Fig. 6). The increase in  $\zeta$ -potential of the reversibly PEGylated polyplexes accompanying the PEG release at acidic pH was also confirmed in this study (Fig. 7). The more rapid release of PEG chains from the polymers at acidic pH compared to neutral pH indicates that the polyplexes would be more stable in extracellular pH, and prompt detachment of PEG chains could occur at the acidic compartments (e.g. endosomes, or lysosomes) inside the cells. The dissociation of PEG chains from the polyplex could increase the exposure of the cationic segments of the polymers to the endosomal membrane, causing membrane disruption and thus further facilitating endosomal escape of the polyplexes. Also, the release of PEG might enable the smoother release of DNA cargo from the polyplexes due to polyanionic exchange with the abundant negatively charged macromolecules present inside the cells. With this environmental sensitivity characteristic, the release of the nucleic acid from the polyplexes after PEG release at acidic pH was expected to be much more efficient. The resulted enhanced transfection by the rPEG polyplexes was shown in Fig. 9. Although the rPEG polyplexes showed one order of magnitude lower in term of transfection efficiency compared to the nPEG polyplexes, they showed more than 1,000 times higher efficiency at every single N/P ratio tested than the irPEG polyplexes, which stably conjugating with the same amount of PEG chains. The *in vitro* transfection experiment also showed that the transfection efficiency of the rPEG polyplexes was similar to that of PEG-*b*-p[Asp(DET)] polyplexes at N/P ratio of 40 and significantly higher at lower N/P ratios. As studies have shown that PEG-*b*-p[Asp(DET)] is a promising *in vitro* and *in vivo* gene delivery agent (21,33), the pH-sensitive multi-PEGylated polymer could also be a valid candidate for future *in vivo* experiments. The success of a non-viral gene delivery vector is also governed by the toxicity caused. It is very common for cationic gene delivery vectors to always possess certain level of cytotoxicity due to their positive charges. The cytotoxicity caused by the polymers synthesized in this study and bPEI was evaluated based on cell metabolism (Fig. 10) and membrane integrity (Fig. 11). In these two studies, bPEI showed significantly higher toxicity compared to the DET-based polymers, which might be a possible reason for low transfection efficiency obtained by high N/P ratio bPEI polyplexes. On the other hand, there was very low toxicity caused by the DET-based polymers even at high N/P ratios or high amine concentrations. One of the advantages of PEGylation of polycationic polymer is to reduce the toxicity (14), but interestingly no reduction of

toxicity could be observed by PEGylation of DET-based polymers in this study. The main reason could be the intrinsic negligible toxicity of DET-based polymers.

## CONCLUSION

In conclusion, a bioresponsive gene delivery vehicle with unique architecture aiming for maintaining a good balance between polyplex stability and polyplex transfection efficiency has been synthesized. The results of the present study show that the PEG attachment through hydrazone linkage to the polymer is pH-sensitive. Majority of the PEG chains can be maintained on the polymer backbone for a long period of time at neutral pH, while the release of PEG is dramatically faster at acidic pH. This pH-sensitive nature allows temporary PEGylation of polyplexes, which can also maintain high transfection efficiency compared to the irreversibly PEGylated counterpart. Furthermore, the physical characteristics and *in vitro* transfection efficiency and toxicity evaluated using the reversibly PEGylated polyplex suggest that it can be a good candidate for future *in vivo* application.

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