

Electronic Supplementary Information

Acetal-linked branched poly(dimethyl-aminoethyl methacrylate) as an acid cleavable gene vector with reduced cytotoxicity†

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Experiments

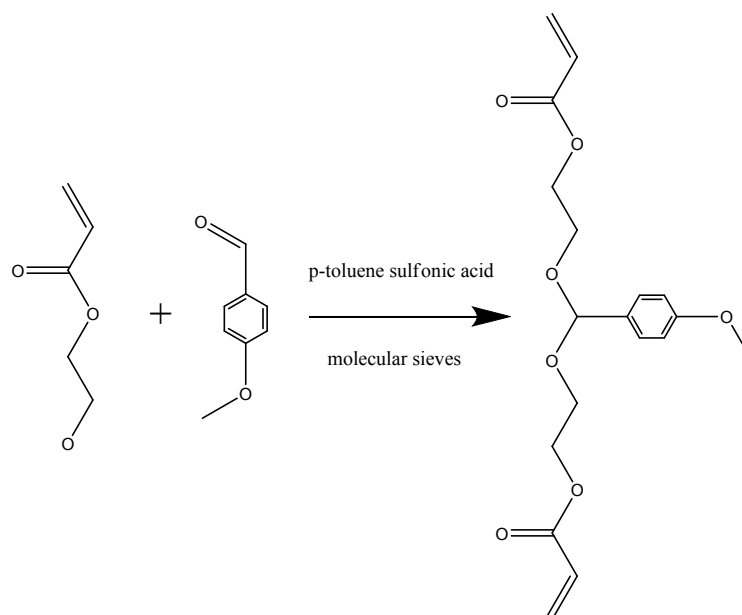
Materials

p-Methoxybenzaldehyde (*p*-MBA) and 2-hydroxyethyl acrylate (HEA) were purchased from Sigma-Aldrich. The *p*-MBA and HEA were dried with a molecular sieve (4 Å) for 24 h before use. Ethyl 2-bromoisobutyrate (EBriB, 98%, Aldrich), pentamethyldiethylenetriamine (PMDETA, 99%, Aldrich), triethylamine (TEA, 99%, Aldrich), *p*-toluene sulfonic acid monohydrate, copper (II) chloride (CuCl₂, 97%, Aldrich), L-ascorbic acid (AA, 99%, Aldrich), ethylene glycol dimethacrylate (EGDMA), and 2-(dimethylamino) ethyl methacrylate (DMAEMA, $M_n=157.22$ g/mol) were used as received.

Preparation and Purification of Di(2-acryloyloxy ethoxy-[4-methoxy-phenyl]methane) Monomer

Di(2-acryloyloxy ethoxy-[4-methoxy-phenyl]methane) for acid cleavable divinyl (named ACD) monomer was prepared by the reported method with a little modification (1). Briefly, the monomers of *p*-MBA (0.1mol) and HEA (0.8mol) were added in a single-necked round bottom flask, with *p*-toluene sulfonic acid (0.015mol) as catalyst and molecular sieves as the drying agent. The mixture was stirred in an ice-water bath overnight and the reaction was stopped by adding 3 ml of TEA. The molecular sieve was removed by filtering under reduced pressure. The reaction mixture was extracted with potassium carbonate aqueous solution (K₂CO₃, 0.1M), then purified by basic alumina column with *n*-hexane/ethyl acetate/TEA mixtures of 75/25/1 as eluents. The obtained product of di(2-acryloyloxy ethoxy)-[4-methoxy-phenyl]methane) was determined by ¹H NMR in CDCl₃: 3.7ppm t (4H), 3.8ppm s

(3H), 4.3ppm t (4H), 5.6ppm s (1H), 5.8ppm d (2H), 6.1ppm q (2H), 6.4ppm d (2H), 7.4ppm d (2H).



Synthesis of Branched Acetal Polymer with Amino-capped Groups

In a typical process for in situ DE-ATRP, the polymers (PDV, with vinyl pendant groups) were synthesized in a 50ml two-necked round bottom flask. DMAEMA (34mmol), bisacrylate acetal monomer (6mmol), EBriB (0.8mmol), 2-butanone (20ml), CuCl_2 (0.2mmol) and PMDETA (0.2mmol) were added to the flask and oxygen was removed by bubbling argon through the solution for 30 minutes. Ascorbic acid solution (0.04mmol AA in 50ul water) was added to the flask by syringe under positive pressure of argon before the flask was immersed in a preheated oil bath at 50°C. The solution was stirred at 800 rpm and polymerization occurred at 50°C in an oil bath for the desired reaction time. Samples were taken from the reaction for analysis at different time points. These were diluted with acetone and dialyzed for 48h in an excess amount of acetone and TEA to purify the polymer.

Distinguished from conventional ATRP that uses a halogen- Cu^{I} /Ligand catalyst, deactivation enhanced ATRP (DE-ATRP) uses a halogen- Cu^{I} /halogen- Cu^{II} mixture which enhances the deactivation of polymerization in the study. Only small amounts of reducing agent, e.g. 5-20% mol L-AA compared to Cu^{II} , were used for producing 10-40% Cu^{I} catalyst instead of 100% Cu^{I} catalyst that in the activator generated by electron transfer (AGET) ATRP. Thus the ATRP deactivation/activation equilibrium

is easily adjusted by the L-AA, which facilitates controlled chain growth and effectively delays the formation of gelation for copolymerization of divinyl monomer.

After removal of solvent, 0.1g pure polymer was re-dissolved into 10ml 0.1M acetone/water (1:1) solution of cysteamine and stirred for 24h and dialyzed against water (pH=8, NaOH water solution) for another 48 h to remove the excess of cysteamine and freeze-dried to get the cysteamine-capped polymer (named PDA, with amino pendant groups). The branched DMAEMA/ethylene glycol dimethacrylate (EGDMA) copolymer (named PD) was synthesized by the same protocol as the PDV except that the bisacrylate acetal monomer was replaced by EGDMA monomer. Similar molecular weight and branched degree were obtained as a control.

Molecular Weight Measurement by Gel Permeation Chromatography

Small samples were withdrawn from the reaction at specific time intervals using a glass syringe with the needle under positive pressure of argon. These were then diluted in DMF and filtered through silica gel for chromatography followed by a 0.2 μ m filter before analysis. Number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (M_w/M_n) were obtained by GPC (920-LC Liquid Chromatograph, Varian) with a refractive index detector and column heater supplied by Varian. The columns (300 \times 7.5 mm PolarGel-M Column, two in series) were eluted using 0.1% LiBr DMF solution and calibrated with poly (methyl methacrylate) standards. All calibrations and analyses were performed at 60 °C and at a flow rate of 1 ml/min.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The polymer was dissolved in CDCl₃ for ¹H NMR analysis and all chemical shifts are reported in ppm relative to TMS. ¹H NMR was carried out on a 400 MHz JEOL NMR with DELTA processing software.

Cell Culture

Rabbit adipose derived stem cells (rADSCs) were grown in Dulbecco modified Eagle's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) at 37°C with 5% CO₂ using standard cell culturing techniques.

Transfection and Cytotoxicity Analysis

Cell secreted Gaussia princeps luciferase plasmid (pCMV-GLuc) was obtained from New England Biolabs UK, and its expansion, isolation and purification was performed using the Giga-Prep (Qiagen) kit as per protocol. The “polyplexes” were formed at various polymer/plasmid (w/w) ratios by mixing the polymer in pure water with the G-luciferase plasmid for 30 minutes prior to use. The electrophoretic mobility of the PDA/DNA complexes at different PDA/DNA ratios were determined by gel electrophoresis using a 7% and 15% agarose gel in a buffer consisting of 45mM Tris-borate and 1 mM EDTA at pH 8.0. Experiments were run at 80V for 90 min. DNA was visualized under UV illumination by staining the gels with Syber[®]Safe DNA gel stain at room temperature.

Under usual cell culture sterile conditions, rADSCs were seeded in 96 well plates at a density of 10,000 cells/well 24 hours prior to the addition of the polyplexes (formed with 1ug of pDNA per well). After the incubation at 37°C and 5% CO₂, the cell culture media (Dulbecco’s Modified Eagles Medium (DMEM) with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin) was replaced with polyplex solutions of varying weight ratio made up in pure water. After 4h of incubation, the media was replaced with serum containing media and incubated for a total of 44h. Control cells were subject to the same treatment, but received plasmid alone. In addition; comparative controls of commercially available transfection agents (PEI, Superfect[™]) were used. For PEI, a ratio of 2:1 was found to be optimal. Superfect[™] was used at a ratio of 10:1 according to manufactures protocol. Analysis of the luciferase activity was performed as per the provided protocol. For secreted luciferase, analysis of the cell supernatant, and subsequent plotting of luciferase activity directly in terms of relative light units (RLU) was done. This renders the cells that received the polyplexes free for cytotoxicity analysis which was performed using the alamarBlue[®] reduction method.

Cell viability was indicated by the reduction of blue alamarBlue[®] solution to a pink color by cell metabolism. This color change is an indicator of cell viability as only live cells can reduce alamarBlue[®] solution so treatment cells are compared with a

positive control of cells under the same conditions but receiving media alone. Absorbance values are then normalized to the control cells (plotted as 100% viable), so any decrease from that of the control cells is a loss of viability. After the incubation time, alamarBlue[®] solution (10% alamarBlue[®] in hanks balanced salt solution (HBS)) was used. The cells are then washed three times with HBS solution before the addition of the alamarBlue[®] solution and a further incubation of three hours were performed. The alamarBlue[®] solution in each well is transferred to a fresh flat bottomed 96-well plate for absorbance measurements at 550nm and 590nm. Viability calculation was followed as per protocol and control cell values normalized to 100% viability. All luciferase and alamarBlue[®] reduction experiments were performed in quadruplicate with margin of error shown as the standard deviation.

Reference

1. Y. Chan, V. Bulmus, M. H. Zareie, F. L. Byrne, L. Barner, M. Kavallaris, *Journal of Controlled Release* **2006**, *115*, 197.

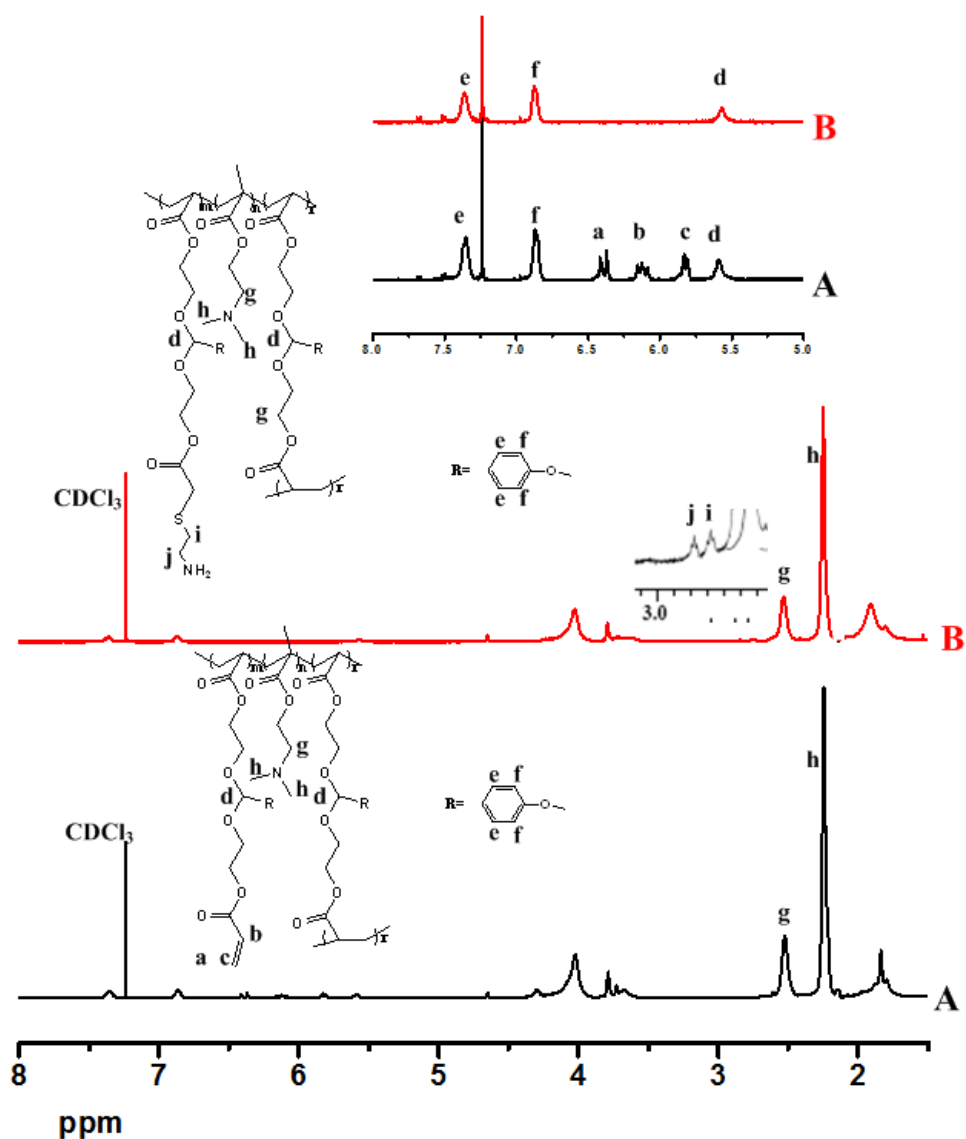
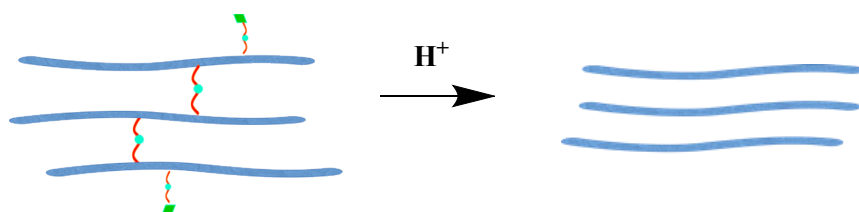
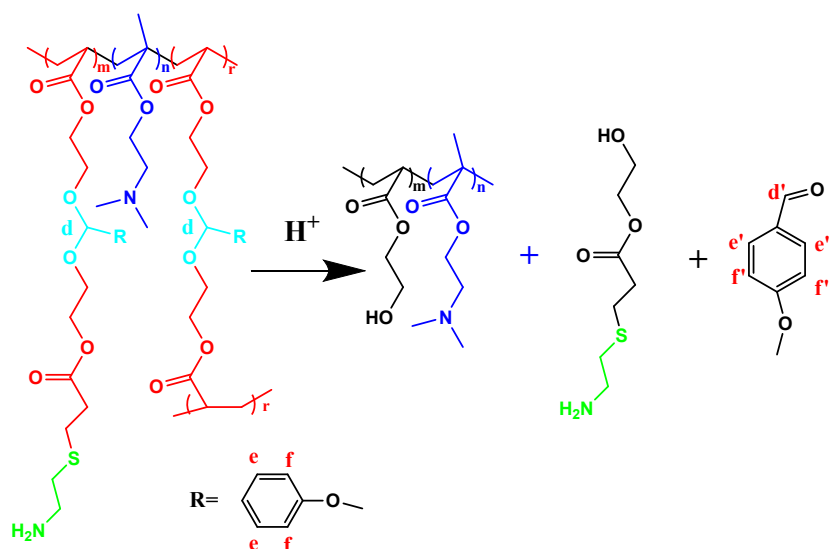


Figure S1 ^1H NMR spectra of the polymer before and after endcapping through Michael addition between the thiol on the cysteamine and vinyl group on the polymer. A, the PDV with vinyl group; B, the PDA polymer with cysteamine end-capped. Top of the right inset is the magnification spectra of counterparts for spectra of A and B.



Scheme S1 the reaction of polymer hydrolyzed to *p*-methoxybenzaldehyde and alcohols compounds at acidic condition, and the scheme for the hydrolysis of branched polymer (bottom).

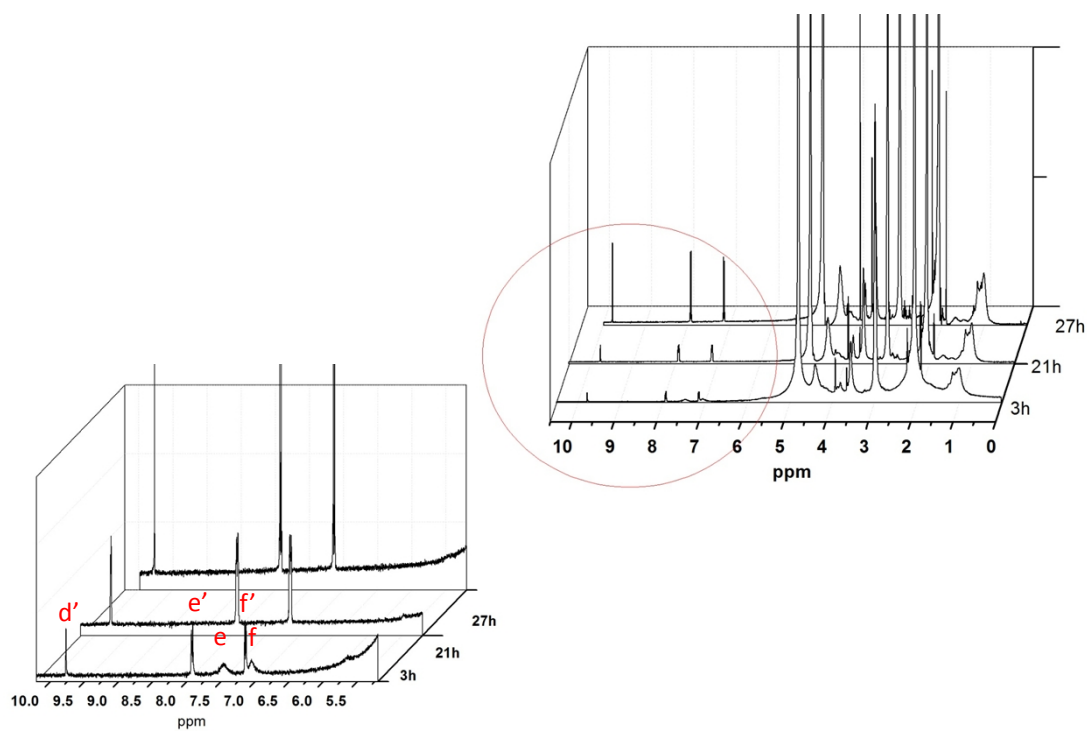


Figure S2 ^1H NMR trace for the hydrolysis of PDA at different time in pH3.

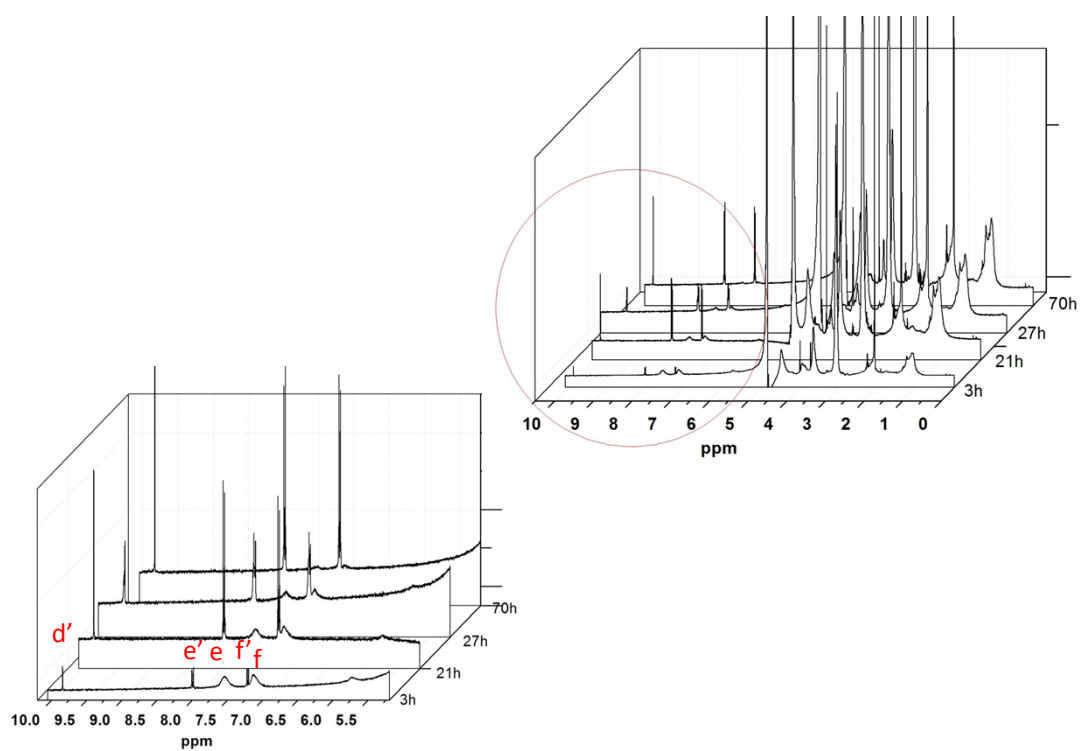


Figure S3 ^1H NMR trace for the hydrolysis of PDA at different time in pH5.

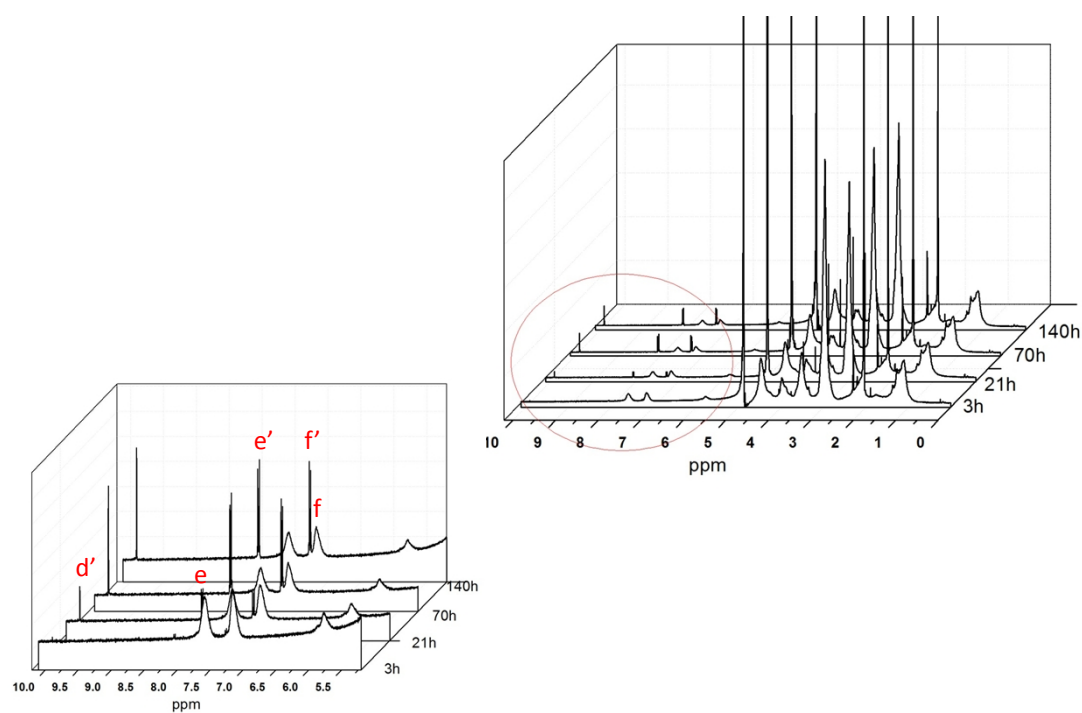


Figure S4 ^1H NMR trace for the hydrolysis of PDA at different time in pH6.

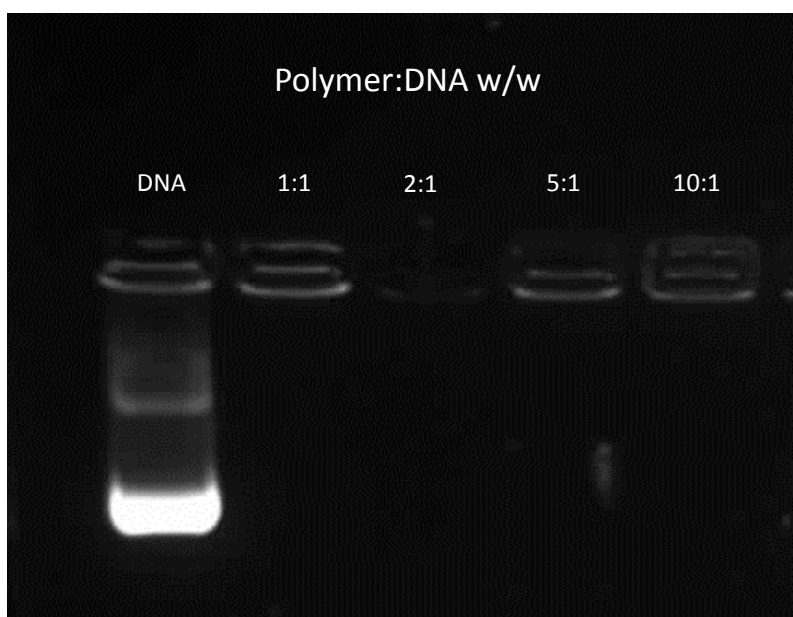


Figure S5 Agarose gel electrophoresis of PDA with plasmid DNA at different ratios of polymer to DNA.

Equation S1

The branching degree, vinyl content and DMAEMA content of the polymer (PDV) were calculated using the following formulae:

$$m = \text{integral of (b)}$$

$$n = \frac{\text{integral of (g)}}{2}$$

$$m + r = \text{integral of (d)}$$

$$\text{DMAEMA content} = \frac{n}{m + n + r}$$

$$\text{Vinyl content} = \frac{m}{m + n + r}$$

$$\text{Branching degree} = \frac{r}{m + n + r}$$

Equation S2

The hydrolyzed degree of the polymer PDA was calculated by the following equation at different pH values according to the ¹H NMR from Fig.S2 to Fig.S4:

$$\text{Hydrolyzed degree} = \frac{\text{integral of (e')}}{\text{integral of (e + e')}}$$