A Highly Effective Gene Delivery Vector - Dendritic Poly(2-(Dimethylamino) Ethyl Methacrylate) From In-situ Deactivation Enhanced ATRP

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Supplementary Information

Materials and Methods

All solvents and reagents were of analytical or HPLC grade and purchased from Sigma or Fisher Scientific unless otherwise stated. Dialysis membrane (MWCO 8000) was used as received from Spectrapor. Ethyl α-bromoisobutyrate (EBr) was used as the radical initiator and the reaction was catalysed by $Copper^{I}$/PMDTA (1,1,4,7,7-Pentamethyl-diethylenetriamine). The molar ratio of DMAEMA to EGDMA was 9:1, with the AA added at 10% of the $CuCl_2$. Dynamic equilibrium was set by the amount of L-ascorbic acid (AA) added to reduce Cu^{II} (deactivated state) to Cu^{I} (propagation state). The reaction was carried out in a 2 neck round bottomed flask under argon, following addition of AA, and carried out for 6 hours at 50°C. Samples were withdrawn at the start and after every hour for GPC (Gel Permeation Chromatography) analysis (Varian 920-LC), with an additional final sample being taken at the end for ^1H NMR (nuclear magnetic resonance) analysis (300 MHz Bruker). Copper was removed from the GPC samples by running through a silica gel column, followed by dilution in DMF (Dimethyl Formaldehyde) for analysis. The reaction was stopped by exposing the solution to the air and the polymer was protected from light throughout the following stages and during storage (-20ºC). The purification process commenced by precipitation in hexane followed by drying under laminar flow. The re-dissolved polymer (in acetone) was reduced to pH 5 by the drop wise addition of 1M hydrochloric acid. This was then dialysed against distilled water for several days, before being freeze dried for subsequent studies. Branched PEI (25kDa) and PLL hydrobromide (40-60kDa) were purchased from Sigma Aldrich. Linear PDMAEMA was synthesised via conventional ATRP method using Cu^{I} catalyst and GPC analysis showed a Mn of (10kDa).

Measurement and Analysis

Gel Permeation Chromatography (GPC)

Molecular weights and molecular weight distributions were determined using a Varian 920-LC instrument with detection performed by the refractive index detector (RI). Chromatograms were run at 40 °C using dimethylformamide (DMF) as eluent with a flowrate of 1 ml/min. The machine was calibrated with linear polystyrene standards. See Table 1 for GPC data.

Spectroscopy

^1H NMR spectra is shown in figure 1. The polymer was dissolved in chloroform and all chemical shifts reported in ppm relative to TMS. The NMR spectrum was used to determine the degree of branching within the polymer structure via the following equations:

$$\text{DMAEMA containing group} = \frac{\text{(integrals of i)/2}}{\text{[integrals of (c+h-i)]/4 + (integrals of i)/2}}$$

$$\text{Vinyl containing group} = \frac{\text{integrals of e}}{\text{[integrals of (c+h-i)]/4 + (integrals of i)/2}}$$

$$\text{EGDMA as branch point} = \frac{[\text{integrals of (c+h-i)}]/4 - (\text{integrals of e})}{[\text{integrals of (c+h-i)}]/4 + (\text{integrals of i})/2}$$

Plasmid purification and polyplex formation

Gaussia Princeps Luciferase (GLuc) plasmid and assay kit (both obtained from New England Biolabs) were used in this research. Plasmid preparation was carried out as explained previously with use of a Maxi-Prep (Qiagen) kit and protocol.[1] Polymer/plasmid solutions were made in phosphate buffered saline (PBS) at various weight ratios by adding 10μg to varying concentrations of polymer. These were left gently shaking for 1 hour to form complexes before analysis. For transfection studies the same technique was used but Dulbecco's Modified Eagles Medium (DMEM) was used instead of PBS. For UV/Vis spectroscopy (NanoDrop™ ND100 Spectrophotometer, Thermo Scientific) 1.5μl of each solution was sampled and repeated 3 times. Graphics shown supporting material are typical spectra (SI figure 4). An agarose gel (10% agarose in Tris-borate-EDTA (TBE) buffer, with SYBR®Safe DNA stain) was made up for all polymers tested. 5μl of each polymer/plasmid solution (DNA concentration of 50 $\mu g ml^{-1}$) were added along with 5μl loading dye to each well and subjected simultaneously to 80mV for up to 2 hours (images inserted in figure 2). For size and charge determination (Malvern Instruments Zetasizer (Nano-2590) solutions of various polymer/plasmid weight ratios were made up as explained above but in serum free media instead of PBS.

Cell culture details

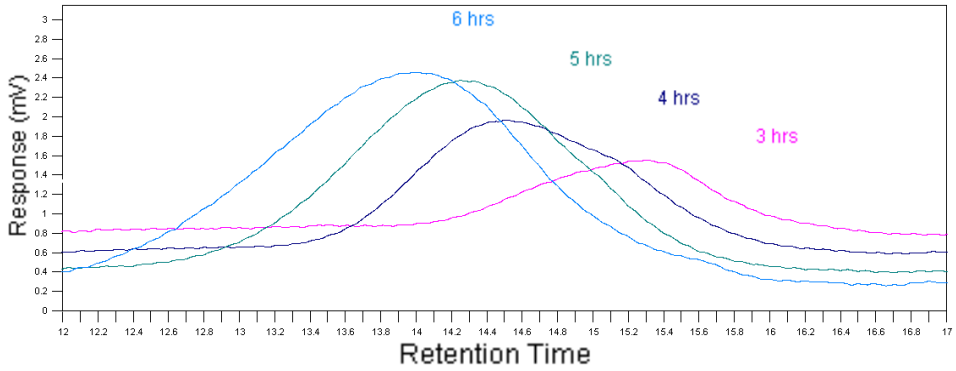
Transfection studies

Under usual cell culture sterile conditions cells were seeded in 96 well plates at a density of 100,000 cells/ml 24 hours prior to the addition of the polymers. After the incubation at 37°C and 5% CO_2 the cell culture media (Dulbecco's Modified Eagles Medium (DMEM) cell media with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin) was replaced with varying weight ratio polyplex solutions made up as described in serum free media. As the transfection ability of polymers changes across cell lines all were tested at a range of weight ratios in a simultaneous study (SI Figure 5). The highest value of each was plotted in the main article.

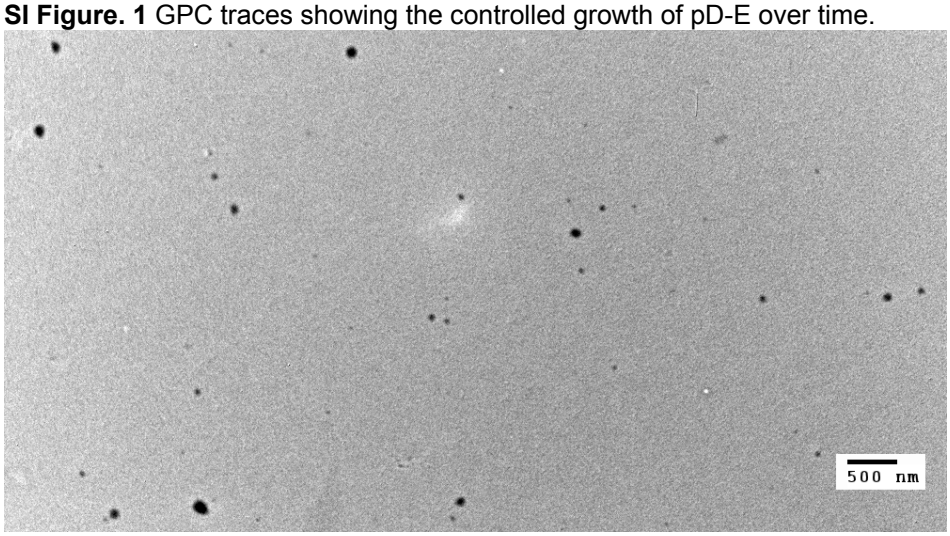
Cytotoxicity analysis

The pD-E polymer along with comparisons (pDMAEMA, PEI, SuperFect® and PLL) were dissolved in cell culture media (DMEM cell media with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin) and the serial dilutions were added to the cells (seeded at 20,000 cells/well of a 96-well plate 24hours prior to the addition of polymer solutions) for the incubation periods at 37°C and 5% CO_2. Cell viability was indicated by the reduction of blue alamarBlue® solution to a pink colour by cell metabolism. Thus 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 is made up of 10% alamarBlue® in hanks balanced salt solution (HBS). The cells are then washed 3 times with HBS solution before the addition of the alamarBlue® solution and a subsequent further incubation of 1 hour. 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 normalised to 100% viability. All values (including standard deviation) were subsequently normalised and plotted (figure 4).

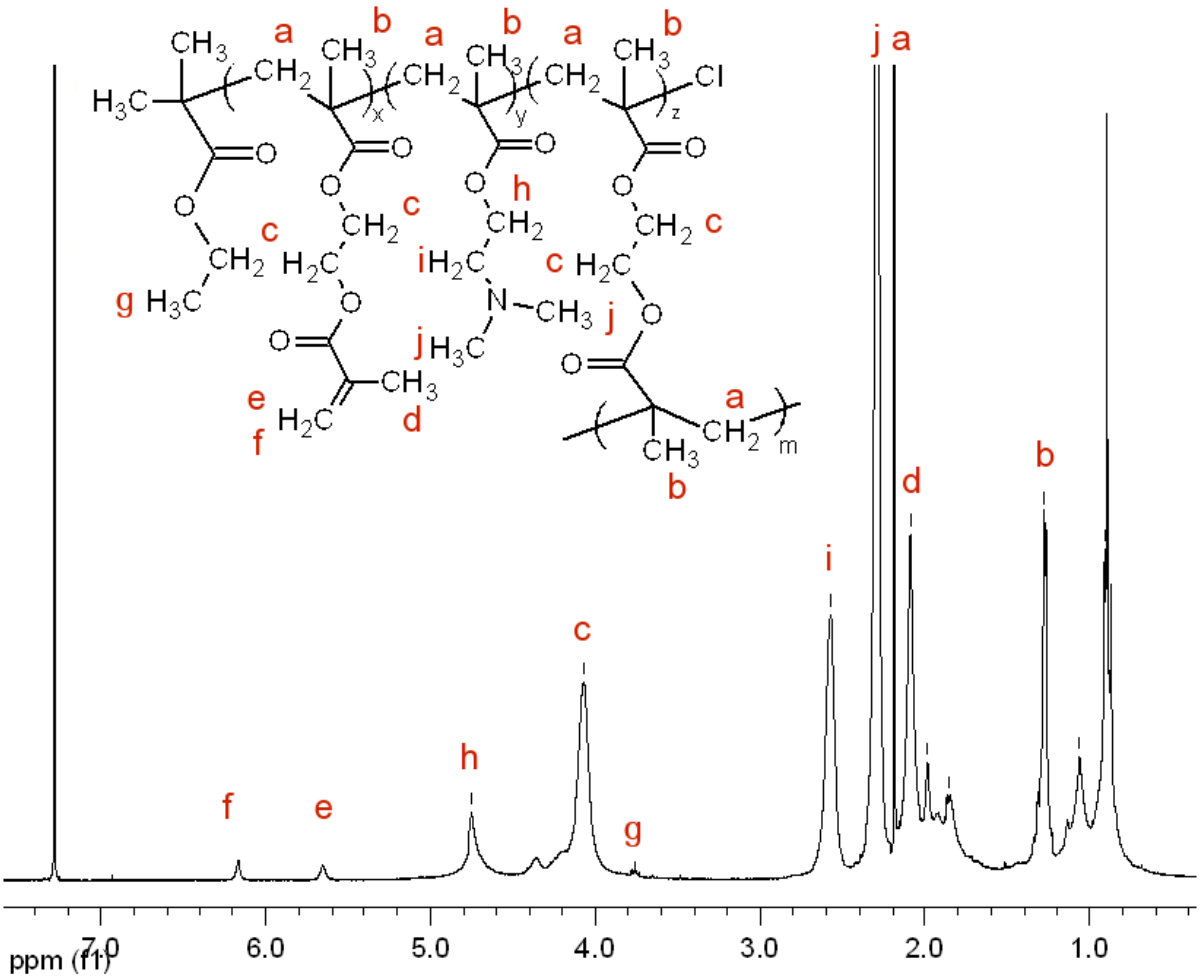
Supplementary Information Figures and Tables



SI Figure. 1 GPC traces showing the controlled growth of pD-E over time.

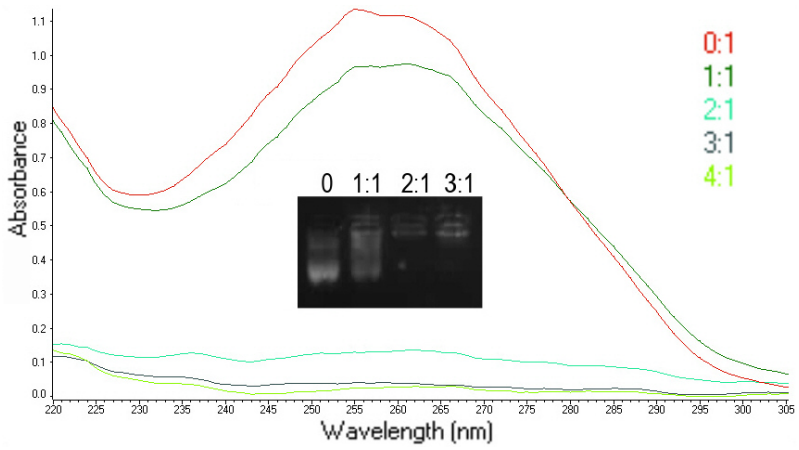


SI Figure. 2 Transmission electron microscope (TEM) (80kV at 10,000x magnification) image of pD-E polyplexes complexed at a 4:1 polymer/plasmid (N/P) ratio.



SI Figure 3. ^{1}H NMR spectrum of pD-E with subsequent assignment of peaks indicated.

a



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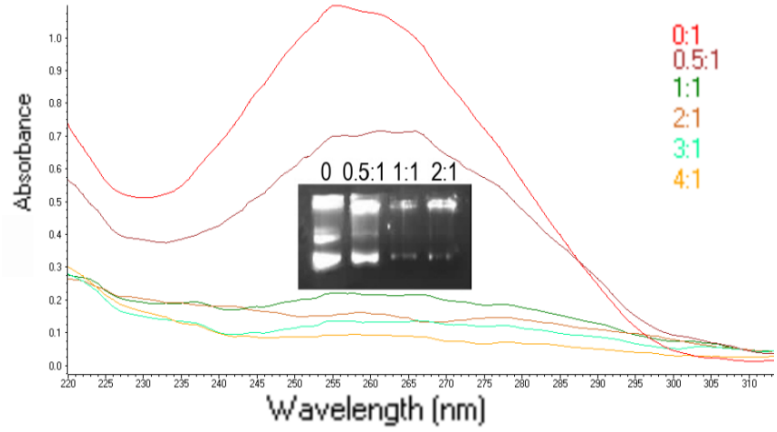

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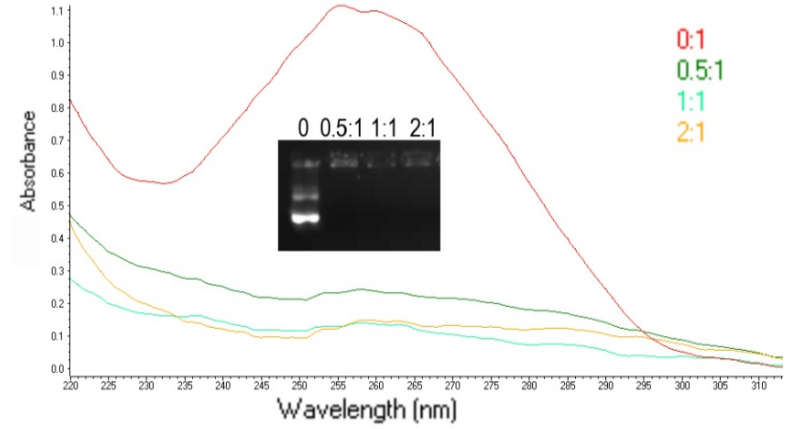
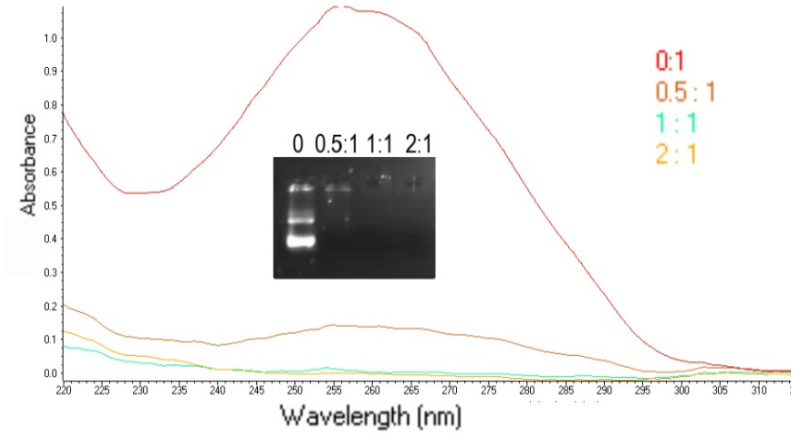
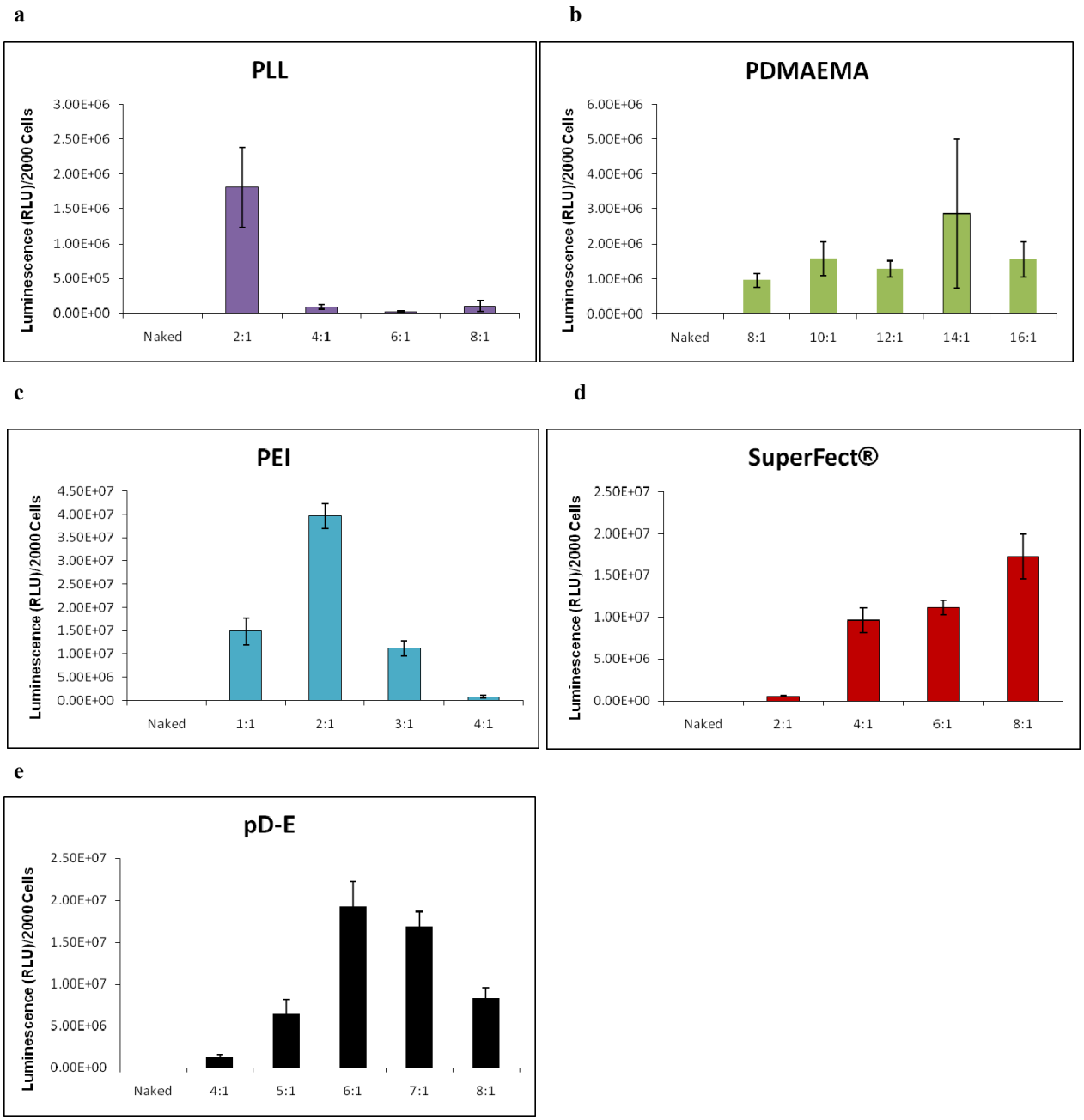


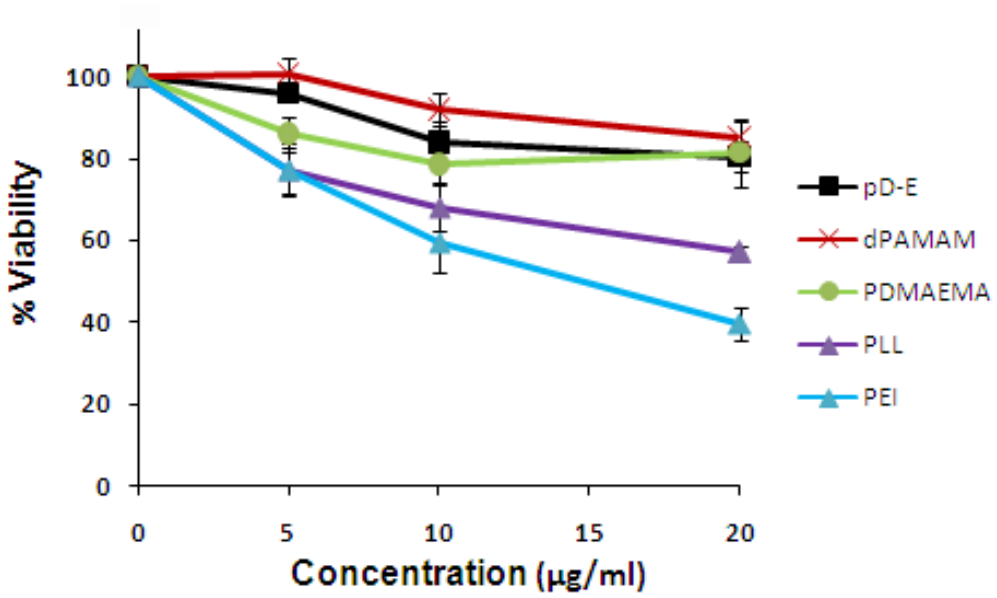
d



SI Figure 4. UV/Vis spectra for samples at various polymer/plasmid weight ratios (indicated) with gel electrophoresis data inset, (a) pD-E, (b) PDMAEMA, (c) PLL, and (d) PEI.



SI Figure 5. a-d show the transfection capability of each polymer at varying polymer/plasmid weight ratios (subsequently N:P ratios calculated for each for quotation in the main text). Values of luminescence for naked plasmid were less than 1000 thus too small to appear (n=4)(±S.D).



SI Figure 6. Viability of 3T3 fibroblast cells after 6hrs incubation with different concentrations of polymers as analysed by the alamarBlue® reduction method. Similar to the results obtained after 24hrs PLL and PEI become significantly more toxic than pD-E at 10µg/ml upward (n=4)(±S.D)($P<0.05$).

[1] C. Holladay, M. Keeney, U. Greiser, M. Murphy, T. O'Brien, A. Pandit, *Journal of Controlled Release* **2009**, *136*, 220.