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

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A hyperbranched 2-(dimethylamino) ethyl methacrylate (DMAEMA) based polymer has been synthesised by a one-pot in-situ deactivation enhanced atom transfer radical polymerisation (DE-ATRP), which exhibits much higher transfection ability than linear poly(DMAEMA) and is comparable to the well known branched poly(ethylene imine) (PEI) and the SuperFect® dendrimer but with lower cytotoxicity.

Gene delivery vectors have technical design criteria such as packaging of large DNA plasmids, protection of DNA, serum stability, specific cell targeting, internalisation, endolysosomal escape and nuclear localisation with minimal toxicity to name a few.1-3 Other criteria include economic and scale-up production factors: ease of administration, ease of fabrication, inexpensive synthesis, facile purification and safety. A much-studied cationic polymer for gene delivery is linear poly(DMAEMA) (PDMAEMA) containing tertiary amine groups for complexing DNA.4,5 When compared with a well-known gene transfer agent poly(L-lysine) (PLL), PDMAEMA shows higher transfection levels and lower toxicity.5 During the drafting of this article, a highly-branched structure of PDMAEMA used for in vitro transfection has been reported.6 However, it only showed a slight increase in transfection efficiency over its linear counterpart, the branched PDMAEMA was still very much overshadowed by the well reported branched PEI standard. Although PEI generally shows high transfection for a non-viral vector, its associated high cytotoxicity is a major drawback and has been a driving force for recent and extensive study into PEI modification.7-9 Dendrimers such as poly(amido amine) (PAMAM) and SuperFect® transfection agent are highly ordered polymers commonly proposed for gene delivery purposes.10 Their well defined branch structures are produced via step by step synthesis methods.11,12 The multistep synthetic processes require extensive purifications after each step, making dendrimer synthesis a costly and time-consuming process.13,14

Here we describe a modification of ATRP (Scheme 1) that allows the formation of a hyperbranched DMAEMA/ethylene glycol dimethacrylate (EGDMA) copolymer (termed as pD-E) in a simple “one pot” reaction, giving the advantages of a branched structure while avoiding the complications of dendrimer synthesis and purification. Unlike conventional ATRP that uses a halogen-Cu/I/Ligand catalyst,15 16 deactivation enhanced ATRP (DE-ATRP) uses a halogen-Cu/I/halogen-CuII mixture which enhances the deactivation of
polymerisation. However in this study, an important difference is introduced, whereby CuCl₂/Ligand is the sole catalytic reagent, but is reduced to Cu⁰ state by a reducing agent L ascorbic acid (L-AA) (scheme 1). Thus the ATRP deactivation/activation equilibrium can be easily adjusted by L-AA ratio, which facilitates controlled chain growth. The gel permeation chromatography (GPC) data (table 1), (see supplementary information for GPC trace), clearly shows the controlled nature of DE-ATRP which delays gelation to allow branching at EGDMA vinyl groups (figure 1). The resulting DMAEMA/EGDMA copolymer was then reduced to pH5 and purified ready for subsequent study (see supplementary information for reaction reagents/conditions/purification and GPC/H NMR spectroscopy characterisation details). Overall, a polymer pD-E (Mn=14kDa, PDI=1.78) with a composition of 78% amine containing groups, 5% EGDMA with vinyl groups and 17% EGDMA used as branching points was formed (see supplementary information). The degree of branching was much higher than the reported SCVP method by Mori et al., and leaves free vinyl groups throughout the structure for further functionalisation with targeting moieties.

The ability of Gaussia Princeps luciferase (G-luc) plasmid DNA to complex with the polymer (pD-E) was assessed by two methods, both using branched PEI (25 kDa), PLL (40-60 kDa), and linear PDMAEMA (10 kDa) as comparisons. Firstly agarose gel electrophoresis was used to test if the plasmids would either remain or be drawn from the well, an indication of complex formation. UV/Vis spectroscopy was also used to determine complex formation via the peak shift mechanism described elsewhere. (See supplementary information for plasmid purification, polyplex formation methods, and analysis method). The insert in figure 2 shows the gel electrophoresis DNA retardation by pD-E at a 2:1 polymer/plasmid N:P ratio (nitrogen/phosphate ratio) indicating good complex formation. This complexing ratio was confirmed via the UV/Vis spectroscopy results (see supplementary information). Polyplex size and charge characterisation was then carried out (see supplementary information for details) as shown in figure 2. At a 2:1 N:P ratio almost neutral polyplexes were formed, but with a larger diameter, possibly due to the polymer bridging different plasmid molecules at this neutral charge. However, further increasing polymer quantity leads to cationic polyplexes forming, resulting in greater condensation of DNA as seen by the reducing size. Transmission electron microscopy was used to visualise these pD-E polyplexes (supplementary information) which, along with zetapotential, UV/Vis spectroscopy and gel electrophoresis, shows the ability of pD-E to condense (load) a typical plasmid at N:P ratios of 2:1 and higher.

The transfection capability of the hyperbranched polymer was then assessed, alongside linear PDMAEMA, PLL, partially degraded PAMAM (dPAMAM) (the SuperFect® transfection reagent) and PEI using fibroblast cells (3T3). Polyplexes were made up in serum free media 1 hour prior to addition to previously seeded cells (see supplementary information for cell culture details and polyplex formation). After 4 hours of polyplex incubation, the media was replaced with fresh serum containing culture media and incubated for a further 44 hrs. Subject to the same conditions, a control group received equal amounts of plasmid but without polymer (termed naked DNA). To analyze transfection the G luc transfection assay kit was used as per protocol. A wide range of ratios were analyzed for each polymer (see supplementary information) and the highest result for each was then compared in figure 3. PEI shows to have the highest transfection capability, with hyperbranched pD-E at an N:P...
This new hyperbranched polymer, via a facile one-pot synthesis, shows potential in the field of gene delivery where further increase in transfection are currently ongoing.

Cell metabolic activity analysis of the polymer (and comparisons) was performed using 3T3 fibroblast cells exposed to three concentrations for two time points, 6hrs (data shown in supporting material) and 24hrs (figure 4). (See supplementary information for details of the experimental method and alamarBlue® assay). The reduction of alamarBlue® solution was used as an indicator of a reduction in viability, for its slightly higher sensitivity than the commonly used MTT assay. Reduced cell viability was seen for all polymers, and at the highest concentration PLL and PEI showed a 50% and 80% reduction respectively. On the other hand, pD-E shows much lower adverse effect on cells, making it a more favorable transfection agent.

In conclusion, the DMAEMA/EGDMA hyperbranched copolymer with highly branched structure, proves to be a highly effective in vitro gene delivery agent. It shows a transfection capability comparable to 25kDa branched PEI, a well-known, highly efficient transfection agent but doesn’t have the drawback of high toxicity. Despite containing less DMAEMA per mass than linear PDMAEMA of similar molecular weight, higher transfection was shown, indicating that the branched structure does significantly affect transfection but less on cellular metabolic activity.

This new hyperbranched polymer, via a facile one-pot synthesis, shows potential in the field of gene delivery where the combination of high transfection coupled with low toxicity is essential.

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References
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-bromoiso-butrylate (EBr) was used as the radical initiator and the reaction was catalysed by Copper^2/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\textsuperscript{II} (deactivated state) to Cu\textsuperscript{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 \textsuperscript{1}H 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 (-20°C). 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\textsuperscript{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

\textsuperscript{1}H 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:

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

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

**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 µg/ml) 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% CO₂ 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% CO₂. 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.
**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 \((n=4)(\pm S.D)(P<0.05)\).