Tailoring Hyperbranched Poly(β-Amino Ester)s: a Synthetic Platform for Epidermal Gene Therapy

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Electronic Supplementary Information

1. Materials and Methods

1.1 Materials

The monomers of trimethylolpropane triacrylate (TMPTA), Bisphenol A ethoxylate diacrylate (BE), 4-Amino-1-butanol (S4), 3-Morpholinopropylamine (MPA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Diethyl ether (99%) was purchased from Fisher Chemical. The plasmids, Gaussia Princeps luciferase plasmid (pCMV-GLuc) and Green Fluorescent Protein plasmid (pCMV-GFP), were obtained from New England Biolabs UK. Poly(ethyleneimine) (branched PEI, 25 kDa) was purchased from Sigma-Aldrich and SuperFect® was purchased from QIAGEN as a positive control for transfection studies.

1.2 Synthesis of highly branched poly(β-amino ester) (HPAE)s

Both HPAE systems, HPAE-A and HPAE-B, were synthesized via Michael addition reaction. The monomer feed ratio of TMPTA:BE:S4 was set as 0.5:1:1.46 in HPAE-A and 1.2:1:2.5 in HPAE-B. For polymerization, monomers were dissolved in DMSO and added into a round bottom flask with a magnetic stirring bar. Monomer concentration in DMSO was 50%. The reaction flask was immersed in an oil bath which was preheated to 90°C. Reactions were terminated when the polymer reached the desired molecular weight, using gel permeation chromatography (GPC) to monitor molecular weight.
Following this, the acrylate terminated based polymer was mixed with 0.12 M end-capping monomer, MPA, in DMSO, and the mixture was stirred for 24 hrs at room temperature. Final product polymers were precipitated in diethyl ether and were then dried under vacuum. Polymers were stored at -20°C as 100 mg/mL solutions in DMSO.

1.3 Gel permeation chromatography (GPC) characterization

Number average molecular weight (Mn), weight average molecular weight (Mw), polydispersity index (PDI), and alpha-value of the Mark-Houwink plots of the polymers were measured by GPC (Agilent Technologies, PL-GPC 50) equipped with a refractive index detector (RI), a viscometer detector (VS DP) and a dual angle light scattering detector (LS 15° and LS 90°). The columns (30 cm PLgel Mixed-C, two in series) were eluted by dimethylformamide (DMF) with 0.1% LiBr at a flow rate of 1 mL/min at 50°C and calibrated with poly(methyl methacrylate) standards (PMMA). All samples were dissolved in DMF at a concentration of 5 mg/ml and passed through a 0.2 \( \mu \text{m} \) filter before analysis.

1.4 Nuclear magnetic resonance (NMR) analysis

\(^{1}\text{H} \text{NMR} \) was performed on a 400MHz Varian NMR system spectrometer and was analyzed using MestReNova processing software. The chemical shifts were referenced to the lock chloroform-d (7.26 ppm).

1.5 Particle size distribution and zeta potential

HPAEs and plasmids were diluted in 25 mM sodium acetate and then mixed at a 1:1 volume ratio with a polymer-to-DNA w/w ratio ranging from 5, 10, to 15. After 10-min incubation, the polyplexes were added to phosphate buffered saline (PBS) in a disposable cuvette. Both particle size distribution and zeta potential were analyzed using a Malvern Zetasizer Nano ZS. Particle size was measured by dynamic light scattering (DLS), and the zeta potential was analyzed by electrophoretic light scattering. The measured range of the particle size was from 0.3 nm to 10 \( \mu \text{m} \) at temperature 25°C and the
measurement for every samples were repeated for four times.

1.6 Agarose gel electrophoresis

Characterization of the polymer/DNA interaction was examined by agarose gel electrophoresis. Agarose gels (1%) containing SYBR® Safe DNA gel stain (Invitrogen) were used to detect the nucleic acids. DNA was diluted to 0.2 μg/μL with sodium acetate. HPAEs were diluted to 5 μL with sodium acetate and then added into the solution of 5 μL DNA over a range of w/w ratio. After 10 minutes, polymer-DNA complex samples and a 6x loading dye, were loaded into each well of the agarose gel. Final volume of each loaded well was 24μL, with gels being run for 25min at 120V.

2. In vitro evaluation

2.1 Cell culture

Four different cell lines were used in this study. Recessive dystrophic epidermolysis bullosa keratinocytes (RDEBK) kindly provided by Dr F. Larcher (Madrid, Spain). Normal human keratinocytes (NHK, HEK001, CRL-2404™) were purchased from ATCC. Both keratinocyte cell lines were cultured in Keratinocyte Growth Medium 2 (PromoCell). All culture media was supplemented with 1% penicillin-streptomycin and culture was maintained in an incubator at 5% CO₂ at 37°C.

2.2 In vitro transfection evaluation

In vitro transfection capability of the HPAE/pDNA polyplexes were assessed via green fluorescence protein (GFP) expression and G-luciferase activity. Cells were seeded at a density of 1x10⁴ cells/well in a 96-well culture plate and grown overnight. Both synthetic polymers and plasmids were diluted in 25 mM sodium acetate (pH 5.2) and then mixed at a 1:1 volume ratio for a polymer-to-DNA w/w ratio of either 5:1, or 10:1. After 10-min incubation, polyplexes were diluted with cell culture medium containing 10% fetal bovine serum (FBS), at a final volume ration of 1:5 polyplex to medium. The final amount of DNA was 0.5 μg/well. After 4-hr incubation, cells were washed, replenished with fresh
medium and incubated for 48 hrs. Transfected cells were analyzed for GFP expression and G-luciferase activity using fluorescence microscope (Olympus) and Gaussia Luciferase Assay Kit (BioLux®), respectively.

2.3 Determination of cell viability

Cell viability was evaluated by alamarBlue™ assay (Thermo scientific), which was considered an index to the metabolic activity of viable cells. Cell viability was assessed 48 hrs post-transfection as described in section 2.2. The alamarBlue™ assay was performed by following the manufacturers’ protocol. After a 2-hr incubation with 10% alamarBlue reagent, absorbance at 570 and 600 nm was recorded on a plate reader (SpectraMax® M3) followed by calculating dye reduction percentage.

2.4 Statistical analysis

G-luciferase activity and cell viability in NHK and RDEBK cells after transfection with HPAE-A2 underwent one-way ANOVA when compared to controls. A $p$-value of $< 0.05$ were considered statistically significant

Reference

Fig. S1 ¹H NMR spectra of HPAE polymer. The peaks at 0.86 ppm and 1.45 ppm are attributed to TMPTA which contains three vinyl groups as Michael acceptor to bind with amino groups to form the highly branched structure. The peaks at 6.78 and 7.11 ppm are another Michael acceptor, BE, which contain two vinyl groups and can act as spacers for extending the length of polymer.

Fig. S2 Plasmid DNA binding ability of HPAE polymers at different w/w ratios (0.5:1 – 8:1).