BEYOND BRANCHING: MULTI-KNOT STRUCTURED POLYMER FOR GENE DELIVERY

*Ahmed Aied[1], Yu Zheng[2], Ben Newland[3], and Wenxin Wang[1]**

[1]Charles Institute of Dermatology, University College Dublin, Dublin, Ireland

[2]The Melville Laboratory, Cambridge University, Cambridge, UK

[3]School of Biosciences, Cardiff University, Cardiff, UK

CORRESPONDING AUTHOR: DR WENXIN WANG, CHARLES INSTITUTE OF DERMATOLOGY, SCHOOL OF MEDICINE AND MEDICAL SCIENCE, UNIVERSITY COLLEGE DUBLIN, DUBLIN, IRELAND. TEL.: +35391493131, FAX.: +353091495585 EMAIL. WENXIN.WANG@UCD.IE

Supporting information

Materials

The monomers 2-(dimethylamino) ethyl methacrylate (DMAEMA) was purchased from Sigma Aldrich. The second monomer, composed of a disulphide linked dimethacrylate (**PEEDEPE**), was synthesized as previously reported by Li *et al*.,[1]. The reagents bis (2-hydroxyethyl) disulfide (BHEDS), triethylamine and methacryloyl chloride for required for synthesis were purchased from Sigma Aldrich. *N,N,N′,N′,N″*-pentamethyldiethylenetriamine (PMDETA, 99%), ethyl 2-bromoisobutyrate (EBriB, 98%), copper(II) chloride ($CuCl_2$, 97%), *d*-Chloroform (99.8%), L-ascorbic acid (AA, 99%) and hydrochloric acid (HCl, 37%) were used as received from Sigma Aldrich. 2-Butanone (HPLC grade, LabScan), tetrahydrofuran (THF, HPLC grade, Fisher), *n*-hexane (ACS reagent grade, Fisher), dichloromethane (ACS reagent grade, Fisher) and dimethylflormamide (DMF, HPLC grade, Fisher) were used as

received. For analysis of the polyplexes, agarose (for electrophoresis, Aldrich), SYBR® Safe Gel stain (Invitrogen), BioLux™ Gaussia Luciferase Assay Kit (New England Biolabs), alamarBlue® (Invitrogen) were used as received and according to manufacturer's protocols.

Methods

PEEDEPE monomer synthesis and purification. All containers used in the reaction were washed with Tetrahydrofuran (THF) before the experiment. THF was first added to the reaction flask with of acryloyl chloride added immediately after. The mixture was cooled down on ice and argon gas bubbled through the stirring solution. While bubbling on ice, triethylamine (TEA) was added slowly. Argon gas was bubbled through the solution for another 10 minutes before the hydroxyethyl disulfide was added dropwise and stirred for 2 hours in the fume hood. The flask was closed and the reaction was allowed to progress for another 24 hours.

Multi-knot polymer synthesis. The polymer was prepared in Acetonitrile (the volume ratios of total monomers to solvent = 1:1) at 60˚C with Schlenk line system, where argon was bubbled through the solutions to remove oxygen. Liquids were transferred by means of septa and syringes while under argon. A typical reaction procedure is described: 2-(Dimethylamino)ethyl methacrylate (DMAEMA) (5.4g, 90 equiv), PEEDEPE (1g, 10 equiv), Ethyl-α-bromoisobutyrate (Initiator) (0.186 g, 2.5 equiv), N,N,N′,N″,N″-

Pentamethyldiethylenetriamine (Ligand) (0.016 g, 0.25 equiv), $CuCl_2$ (0.013 g, 0.25 equiv) and acetonitrile (7ml, 50%w/w) were transferred to a two necked round-bottom flask fitted with stopcocks. Argon was bubbled through the solution for 15 minutes to purge the oxygen. L-ascorbic acid (0.0034g, 0.05 equiv) was added into the flask to start the reaction, which was kept in an oil bath at 60˚C and stirring at 600rpm. The reaction was stopped when the desired molecular weight of polymer was obtained.

Polymer purification. After polymerization, the polymer was precipitated by adding the solution drop-wise into a large excess of hexane and diethyl ether (1:1) to remove excess DMAEMA and PEEDEPE monomers. The precipitated polymer was dissolved in acetone and passed through an aluminum oxide glass column to remove the copper. Acetone was removed by rotary evaporation and the polymer was dissolved in water for the next procedure.

Conjugate addition of diamine monomers and protonation. Unreacted vinyl groups of polymer were end-capped by adding 200mg (2mmol, dissolved in water) of the polymer to 40mg (50mmol, dissolved in water) of either 1,3-Diaminopropane or ethylenediamine under argon at ambient temperature for 48 hours in the dark. The end-capped polymer solution was protonated to pH 5.5 by adding 1M HCL dropwise under stirring while constantly monitoring the pH using a pH meter (FiveEasy^{TM}, Mettler Toledo). Finally, the solution was freeze-dried and a white soft sponge was obtained.

Gel Permeation Chromatography (GPC).

Samples were taken at different time points and then diluted in dimethylformamide (DMF) and filtered through an Al_2O_3 pipette for chromatography followed by a 0.2μm filter before analysis. The molecular weight and molecular weight distribution of each sample was determined using a Varian 920-LC instrument with a refractive index detector (RI).

Chromatograms were run at 60 °C using DMF as eluent with a flow rate of 1 ml/min. The machine was calibrated with linear poly (methyl methacrylate) standards.

Nuclear magnetic resonance (NMR) spectroscopy.

Proton NMR analysis was carried out on a S4 300 MHz Bruker NMR, and the chemical shifts were referenced to the lock chloroform (7.26 p.p.m.).

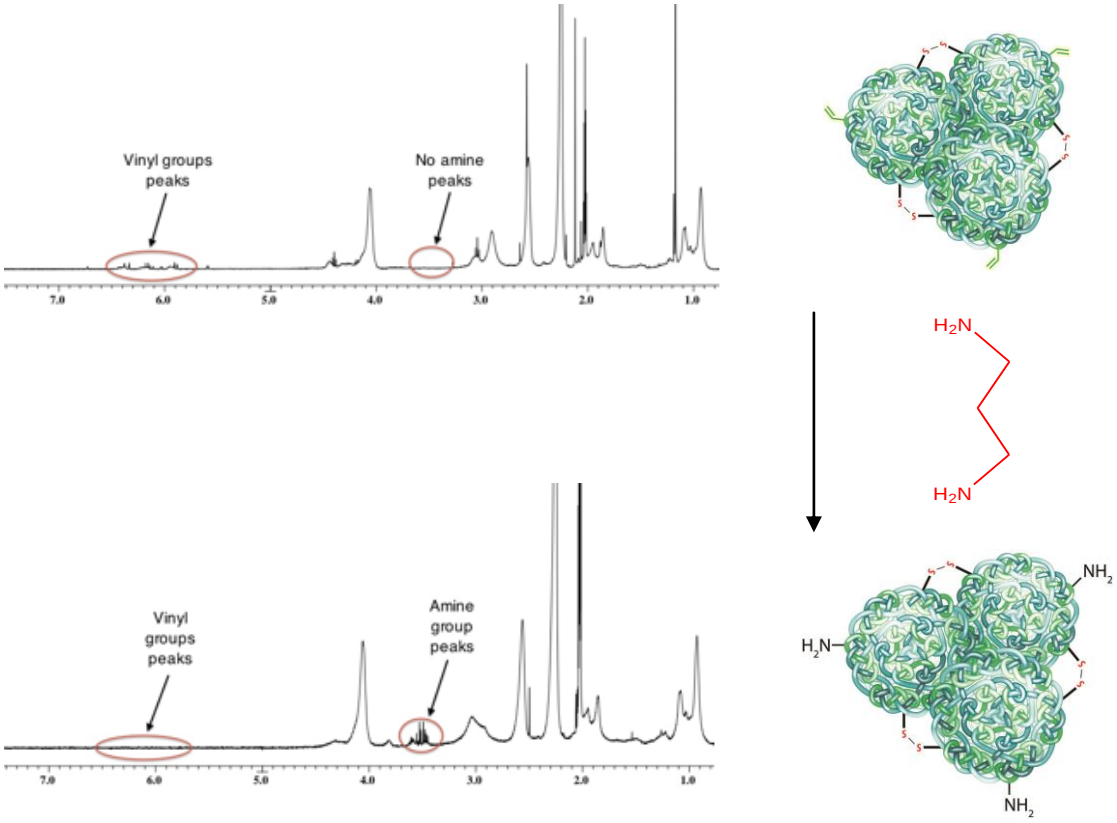

Figure S1: Proton NMR spectra of the multi-knot polymer before (top) and after (bottom) Michael addition of diaminopropane.

Transfection analysis. *Gaussia* luciferase expression and cell metabolic activity analysis of the multi-knot yielded insight into the polymer's capability to efficiently deliver and release DNA into cells without exhibiting cytotoxicity. The protein expression results indicate that the polymer is capable of efficiently delivering the luciferase plasmid to adipose derived stem cells (ADSCs) and fibroblasts. A range of polymer to DNA weight ratios (w/w)

were examined for all cell types and it was discovered that the optimal weight ratio differed for each cell type. Fibroblasts showed maximum luciferase expression at 10:1, while ADSCs showed better expression with a five times lower ratio (2:1). We saw significantly higher luciferase activity in ADSCs transfected with the multi-knot polymer compared with cells transfected with PEI. We previously showed higher levels of green fluorescent protein (GFP) expression in 3T3 fibroblasts transfected with single knot polymer and lower cytotoxicity compared to PEI[2]. It is clear from the data that transfection efficiency is different for each cell type. For example, protein expression levels in ADSCs transfected with the multi-knot were 3 fold higher than cells transfected with PEI. This trend is repeated in fibroblasts but with no statistical significant difference ($n=3$, $P<0.05$). In addition, the overall reporter gene expression is ~10-fold lower in the fibroblasts compared with ADSCs, indicating that this cell type is harder to transfect. The cell metabolic activity of all cell types transfected with the multi-knot polymer is significantly higher than PEI with more than 80% viability.

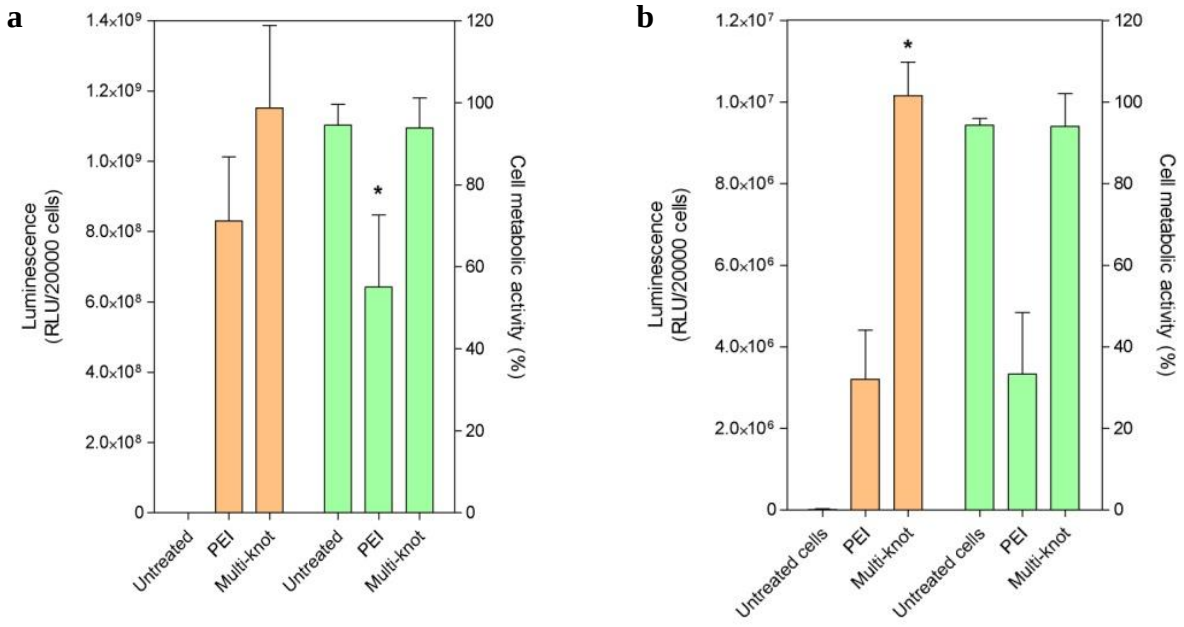

Figure S2: Reporter gene delivery efficiency and cytotoxicity of the multi-knot polymer. *Gaussia* luciferase was used as the reporter plasmid for transfection of **a**. RDEB fibroblasts and **b**. ADSCs. Cytotoxicity was determined from cell metabolic activity measurements using a cell health indicator. Error bars represent upper and lower values of standard deviation and asterisks represent significant difference between PEI and multi-knot polymer expressed as mean ±S.D. (*n*=3). The one way ANOVA was used for statistical analysis.

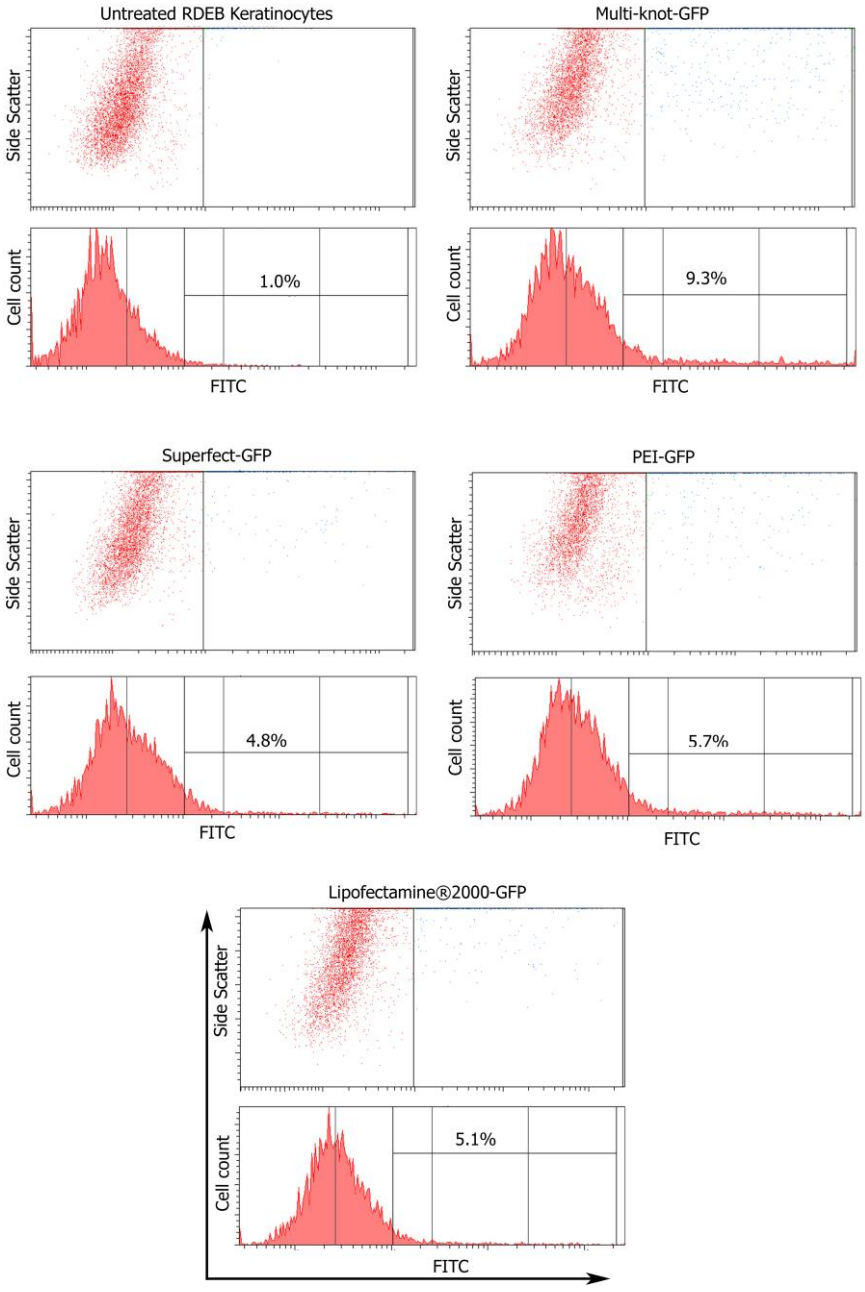

Figure S3: Scatter plots of total live cells and GFP+ cells. RDEB keratinocytes were transfected with multi-knot polymer and commercial agents. Propidium Iodide was used to identify the live cells using the PI channel while GFP was detected using the FITC channel.

Standard protein expression analysis procedures. For immunofluorescence analysis of monolayer keratinocyte cultures, cells were seeded on enclosed glass slides ($1x10^4$cells/well) and incubated at 37°C for 48 hours post transfection. Cells were permeabilized and fixed in cold paraformaldehyde, fixed with 0.5% bovine serum albumin and incubated for 1 hour with the monoclonal antibody LH7.2 (C6805 Sigma Aldrich) derived from mouse and directed against human type VII collagen diluted at 1:1000 in PBST/3% BSA. This antibody recognizes an epitope on the NC-2 carboxy terminal region and so confirms the expression of the recombinant type VII collagen. Alexa-Fluor488 donkey antimouse IgG (Invitrogen) was used as the secondary diluted to 1:1000 in PBS. Cells were counterstained with Rhodamine-phalloidin and 4, 6-diamidino-2-phenylindole (DAPI) at 1:500 and 1:200 dilutions respectively. Images were taken with 20x magnification using an Olympus IX81 inverted microscope. Image analysis was carried out using ImageJ 1.44p (Wayne Rasband, National institutes of Health, USA).

For western blot analysis, keratinocytes were maintained in T25 flasks and fed with growth medium. The supernatant containing the excreted protein type VII collagen was collected and concentrated using Amicon Ultra-15 centrifugal filters. Equal amounts of total protein (20μg) were subjected to denaturing 4-15% gradient SDS-PAGE and transferred to a nitrocellulose membrane (Whatman® PROTRAN BA 85 NITROCELLULOSE, Sigma-Aldrich) by

electro-blotting according to standard procedures. For type VII collagen, blots were reacted with the monoclonal antibody used above at 1:1000 dilution in blocking buffer (3%BSA in TBS-Tween20) overnight. After washing, the secondary goat anti-mouse IgG-HRP was added to the membrane and incubated at room temperature for 1 hour prior to visualization using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific imaged with Mason technology, Syngene G:Box, and Synoptics Ltd. GeneSnap software).

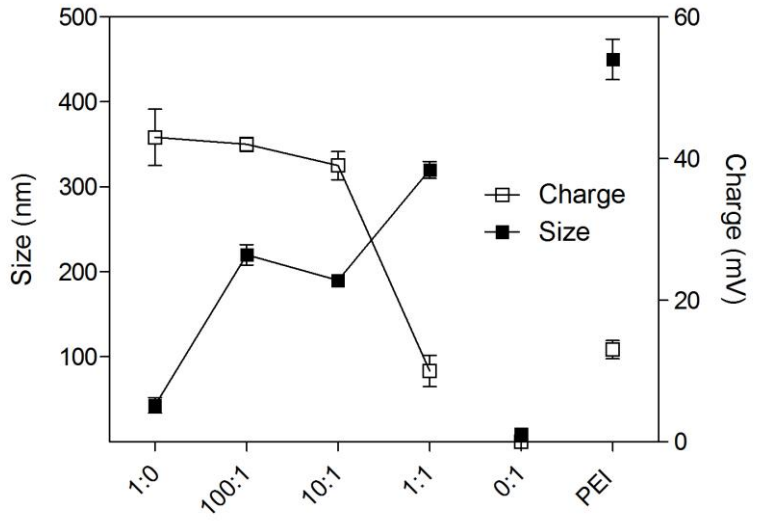

Figure S4: Average charge and average size of nanoparticles formed by the multi-knot polymer with luciferase plasmid (polymer : plasmid) at different ratios in Dulbecco's Modified Eagle Medium.

SI References

1. Li, Y.; Armes, S. P. Synthesis and chemical degradation of branched vinyl polymers prepared via ATRP: use of a cleavable disulfide-based branching agent. Macromolecules 2005, 38, 8155-8162.

2. Newland, B.; Zheng, Y.; Jin, Y.; Abu-Rub, M.; Cao, H.; Wang, W.; Pandit, A. Single cyclized molecule versus single branched molecule: a simple and efficient 3D "knot" polymer structure for nonviral gene delivery. J Am Chem Soc 2012, 134, 4782-9.