

## **A knot polymer mediated non-viral gene transfection for skin cells**

Lara Cutlar,<sup>a,⊗</sup> Yongsheng Gao,<sup>a,⊗</sup> Ahmed Aied,<sup>a,⊗</sup> Udo Greiser,<sup>a</sup> Eva Maria Muraier,<sup>b</sup> Dezhong Zhou,<sup>a,\*</sup> and Wenxin Wang<sup>a,\*</sup>

<sup>a</sup>Charles Institute of Dermatology, University College Dublin, Dublin 4, Ireland

<sup>b</sup>Division of Molecular Dermatology and EB House Austria, Department of Dermatology, Paracelsus Medical University, Salzburg, Austria

<sup>⊗</sup>These authors contributed equally to this work.

\*Corresponding authors: [dezhong.zhou@ucd.ie](mailto:dezhong.zhou@ucd.ie) and [wenxin.wang@ucd.ie](mailto:wenxin.wang@ucd.ie)

## **Materials and Methods**

### **Materials**

Bis(2-acryloyl)oxyethyl disulphide (BAD) monomer was synthesized according to the literature<sup>[1]</sup>. 2-(dimethylamino)ethyl methacrylate (DMAEMA), Ethyl- $\alpha$ -bromoisobutyrate, N,N,N',N'',N''-pentamethyldiethylenetriamine, CuCl<sub>2</sub>, acetonitrile, L-ascorbic acid, ethylenediamine and aluminium oxide and chloroform-d were purchased from Sigma. Hexane, diethyl ether, dimethylformamide and acetone were purchased from Fisher. All chemicals were used as received.

### **Synthesis of DSP**

The poly (2-dimethylaminoethyl methacrylate-co-bis(2-acryloyl)oxyethyl disulphide) was synthesized by DE-ATRP. A typical reaction procedure is described here: 2-(dimethylamino)ethyl methacrylate (DMAEMA) (5.4 g, 90 equiv), BAD (1 g, 10 equiv), Ethyl- $\alpha$ -bromoisobutyrate (0.186 g, 2.5 equiv), N,N,N',N'',N''-pentamethyldiethylenetriamine (0.016 g, 0.25 equiv), CuCl<sub>2</sub> (0.013 g, 0.25 equiv) and acetonitrile (7 ml, 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 (0.0034 g, 0.05 equiv) was added into the flask with stirring at 600 rpm to start the reaction at 60°C in an oil bath. The reaction was stopped when the desired molecular weight of the polymer was obtained. The obtained polymer was purified by precipitation in hexane and diethyl ether (1:1) to remove excess DMAEMA monomers and PEEDEPE. The precipitated polymer was

dissolved in acetone and passed through an aluminium oxide glass column to remove the copper. Acetone was removed by rotary evaporation. Then, unreacted vinyl groups in the polymer were end-capped by adding 200 mg (2 mmol, dissolved in water) of the polymer to 40 mg (50 mmol, dissolved in water) of ethylenediamine under argon at ambient temperature for 48 hours in dark. The end-capped polymer solution was protonated to pH 5.5 by adding 1M HCL dropwise, freeze-dried and a white soft sponge was obtained. Then, the polymer was dialyzed with deionized water for three days to remove the unreacted ethylenediamine and freeze dried.

### **Gel Permeation Chromatography (GPC)**

The molecular weight of the polymer was monitored with GPC during the polymerization. Samples were taken at different time points, diluted in dimethylformamide (DMF) and filtered through an Al<sub>2</sub>O<sub>3</sub> pipette for chromatography followed by a 0.2 µm 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**

Chemical structure and composition of DSP was verified by NMR, which was carried out on a 300 MHz Bruker NMR, and the chemical shifts were referenced to the lock

chloroform (7.26 p.p.m.). NMR results of DSP:  $^1\text{H}$  NMR:  $^1\text{H}$  NMR: 0.7-1.2 (m,  $\text{CH}_3\text{CH}_2-$ ,  $-\text{C}(\text{CH}_3)_2$ ,  $-\text{CCH}_3$ ), 1.7-1.9 (m,  $-\text{CH}_2\text{CH}-$ ,  $-\text{CCH}_2-$ ), 2.2-2.4 (m,  $-\text{N}(\text{CH}_2)$ ,  $-\text{CHCH}_2-$ ), 2.4-2.6 (m,  $\text{NH}_2\text{CH}_2\text{CH}_2\text{NH}-$ ), 2.6-2.8 (m,  $-\text{CH}_2\text{N}(\text{CH}_2)_2$ ), 2.9-3.0 (m,  $-\text{SCH}_2-$ ), 3.3-3.5 (m,  $-\text{NHCHCOO}-$ ), 4.0-4.5 (m,  $-\text{NCH}_2\text{CH}_2\text{O}-$ ,  $-\text{SCH}_2\text{CH}_2\text{O}-$  and  $-\text{CHBr}$ ).

### **Production of plasmid DNA**

COL7A1 DNA of 8.9kb (subcloned into pcDNA 3.1) was obtained from Dr Andrew South (Surgery and Molecular Oncology Department, University of Dundee, Ninewells, Dundee, UK) XL-10 Ultracompetent Gold E-coli (Stratagene, USA) were transformed and grown in LB media supplemented with 100ug/ml ampicillin for positive selection. The COL7A1 plasmid (14.3 kb) was purified by endotoxin-free Giga-plasmid preparation kit (QAIGEN, West Sussex, UK).

### **Polyplex preparation**

To prepare polyplexes, DSP and DNA (Gaussia luciferase and COL7A1) were dissolved in distilled water at 1 mg/ml and 0.1 mg/ml. Then, according to weight ratios, different volumes of DSP solution were added into the DNA solution, mixed for 10 seconds in a vortex and then allowed to stand for another 15 minutes to form complex. For the commercial gene transfection reagents PEI and Lipofectamine 2000, the polyplexes were formulated as per the manufacturers' protocols.

## **Polyplex characterisation**

DNA binding affinity of the DSP was first evaluated with Picogreen assay<sup>[2]</sup>. Briefly, the polyplexes were prepared as above. Then, Picogreen solution was added and incubated for another five minutes. Afterwards, the polyplex solution was added into 200  $\mu$ l of DMEM in a black 96 well plate, the fluorescence was read in a plate reader at a 490 nm excitation and a 535 nm emission. As size and charge are the leading factors for cell uptake, hydrodynamic diameters and zeta ( $\zeta$ ) potentials of a range of cyclized DSP/DNA complexes were measured by light scattering and zeta potential analyser (Malvern instruments, Zetasizer Nano-ZS90). Polyplex solutions (1 ml) containing 10  $\mu$ g of DNA were prepared at various weight ratios. Sizes and  $\zeta$ -potential are presented in the results as the average values of 5 runs.

## **Cell culture**

HeLa and HEK 293 cells ordered from ATCC, UK were cultured with DMEM with 10% FBS and 1% P/S under standard conditions (37°C, 5% CO<sub>2</sub>). The type VII collagen null-RDEB keratinocytes-RDEB-TA4 (RDEBK) and type VII collagen null-RDEB fibroblasts (RDEBF) were kindly provided by Dr F. Larcher (Madrid) and A. Klausegger (Salzburg), respectively. The RDEBK were harvested from a patient with a homozygous mutation 6527insC => TAA Stop 337 bp downstream (2176) in exon 80 of COL7A1 and were immortalized by SV40 Large T Antigen.<sup>[3]</sup> The RDEBF are homozygous for 706C>T in exon 6 of the COL7A1 gene and were immortalized with HPV-16 E6/E7 oncogene. RDEBK were cultured in Basal media with Keratinocyte

Growth Medium 2 Supplement Pack (Promocell). RDEBF were cultured in DMEM:F-HAM's12 (1:1) (Sigma-Aldrich) and 10% Fetal Bovine Serum (Sigma-Aldrich).

### **Gene transfection**

HeLa and HEK 293 cells were seeded in 96 well plates at a density of 20000 cells per well one day prior to transfection and cultured until 70~80% confluence. Polyplexes were prepared as mentioned above. The growth media in the wells was removed and then cell culture DMEM containing polyplexes was added (0.5 µg DNA per well was used). After 4 hours of incubation, the polyplexes were removed and fresh complete growth media was added. The cells were cultured for another 44 hours before measuring the transfection efficiency by luminescence intensity according to the manufacturer's protocol (BioLux® Gaussia luciferase assay kit, New England BioLabs®, UK). For flow cytometry measurement, HeLa cells were seeded in 24 well plates at a density of 100000 cells per well and 2 µg GFP DNA per well was used. Transfection was carried out as mentioned above. After 48 hours, cells were collected as per standard protocols and then propidium iodide was used to exclude the dead cells. At least 10000 cells were counted. The transfection of RDEB keratinocytes and fibroblasts was carried out in 24 well plates. Cells were seeded at a density of 10000 cells per well. Polyplexes were prepared as above. The growth media was removed from the cells. 500 µl of DMEM containing the polyplexes was then added. After 4 hours of incubation, the polyplexes were removed and fresh complete growth media

was added. Twenty-four hours after transfection, ascorbic acid (50 µg/ml) was added. Cells were fixed 48 hours after transfection for immunofluorescence.

### **Metabolic activity**

The cell metabolic activity was measured after 4 hour incubation with the polyplexes using the alamarBlue® assay (Invitrogen). 10 µl /well of alamarBlue® was added to transfected and untreated (control) cells in 90µl/well of media and incubated at 37 °C for 4 hours. Microplate (Thermo Scientific, Varioskanflash multimode reader) fluorescence measurements were then taken at 560EX nm/590EM nm filter settings. Results were obtained as the mean and standard deviation from triplicate values and displayed as a percentage relative to untreated control cells.

### **Immunofluorescence staining**

For immunofluorescence analysis of monolayer keratinocyte cultures, cells were seeded on enclosed glass slides (1x10<sup>4</sup>cells/well) and incubated at 37 °C for 48 hours post transfection. Cells were permeabilized and fixed in cold paraformaldehyde and incubated for 1 hour with the anti-type VII collagen monoclonal antibody LH7.2 at 1:1000 in PBST/3% BSA. The anti-mouse Alexa-Fluor488 donkey secondary antibody (Invitrogen) was 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).

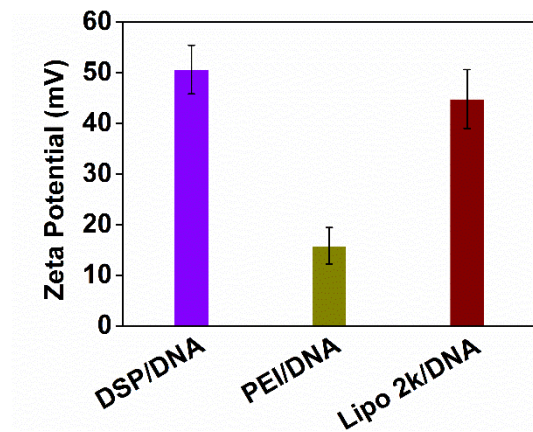
### **Western Blotting**

Both RDEB and wild type keratinocytes were cultured for 2 days in medium as above supplemented with ascorbic acid (50 µg/ml). The cells were pelleted at 2000 rpm and then lysed with a chilled RIPA buffer containing Protease inhibitor cocktail. Concentration of cell lysates was determined with Bradford assay and ~120 µg aliquot of each lysate was resolved on a 4-20% SDS-polyacrylamide gel. As a loading control the gel was then cut in the horizontal for detection of β-actin. The fractionated proteins were transferred to nitrocellulose transfer membrane overnight at 18 mA. The membranes were then blocked with 5% BSA-TBS for 2 hours at room temperature and incubated for another 2 hours with a rabbit polyclonal antibody against the collagen VII NC1 domain (1/2,000) and a murine antibody for β-actin detection (1/2,000). The secondary antibodies HRP fluorescent was visualized by enhanced chemiluminescence according to the manufacturer's instructions. The exposure time was 5 minutes for all samples.

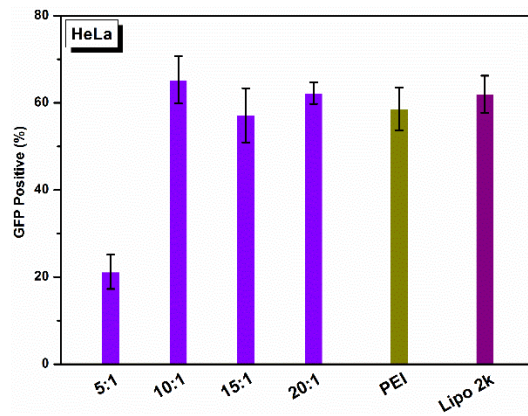
- [1] A. Aied, Y. Zheng, B. Newland, W. Wang, *Biomacromolecules* **2014**, *15*, 4520-4527.
- [2] J. J. Green, G. T. Zugates, N. C. Tedford, Y. H. Huang, L. G. Griffith, D. A. Lauffenburger, J. A. Sawicki, R. Langer, D. G. Anderson, *Advanced Materials* **2007**, *19*, 2836-2842.
- [3] C. Chamorro, D. Almarza, B. Duarte, S. G. Llames, R. Murillas, M. Garcia, J. C. Cigudosa, L. Espinosa-Hevia, M. J. Escamez, A. Mencia, A. Meana, R. Garcia-Escudero, R. Moro, C. J. Conti, M. Del Rio, F. Larcher, *Experimental dermatology* **2013**, *22*, 601-603.



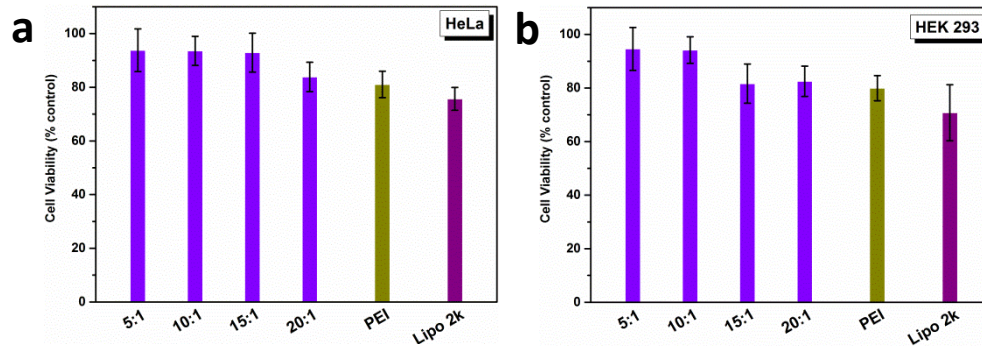
## Figures



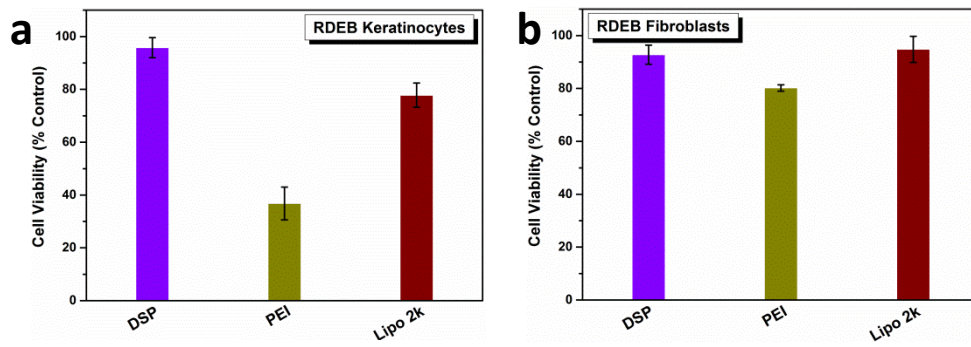
**Figure S1.** At w/w=10:1, DSP can condense DNA into polyplexes with a zeta potential around +50 mV. Polyplexes were also formed with PEI (15mV) and Lipofectamine 2k (45mV). Sample measurements were taken at n=3.



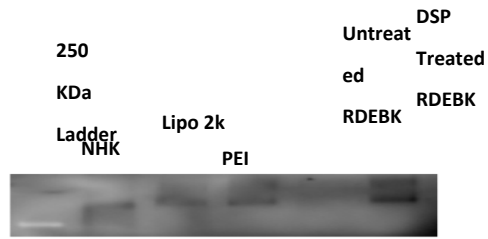
**Figure S2 GFP positive of HeLa cells after transfection measured by flow cytometry. The levels of GFP expression are measured across different ratios of DSP to DNA, (5:1, 10:1, 15:1 and 20:1) and compared to those reached with PEI and Lipofectamine 2k. All cell samples (excluding the 5:1 ratio of DSP) were found to be in the region of 60%, samples were measured at n=3.**



**Figure S3. Cell viability after treatment with DSP at various w/w ratios, PEI or Lipofectamine 2000.**



**Figure S4. Cell viability after treatment with DSP at w/w=10:1, PEI or Lipofectamine 2000.**



**Figure S5. The rabbit polyclonal antibody against the collagen VII was used for western blotting. A 290 KDa band of type VII collagen was detected for Lipofectamine 2000, PEI and DSP treated cells.**