**ELECTRONIC SUPPORTING INFORMATION**

# Solid-Phase Synthesis of a Lysine-Capped *bis*-Dendron with Remarkable DNA Delivery Abilities

*Siew-Eng How, Asier Unciti-Broceta, Rosario M. Sánchez-Martín, and Mark Bradley**

University of Edinburgh

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## 1. Experimental

### 1.1. General information

$^{1}H$ and $^{13}C$-NMR spectra were recorded on a Bruker DPX-400 (400 and 100 MHz, respectively) and a Bruker AC-300 (300 and 75 MHz, respectively) in the solvents indicated at 298 K. Chemical shifts for proton and carbon spectra were reported on the δ scale in ppm and were referenced to residual solvent. All coupling constants (*J* values) were measured in Hz. ES and EI mass spectra were recorded using a VG Platform Quadrupole Electrospray Ionisation mass spectrometer and ThermoQuest TraceMS single quadrupole GC-MS respectively. Infra red spectra were recorded using a Bio-Rad FT-IR spectrometer Paragon 1000 with a golden gate accessory with neat compounds. Thin layer chromatography (TLC) was performed using Alugram SIL G/UV/254 precoated plates and visualised by ultra-violet light and/or stained with ninhydrin (0.3% ninhydrin in *n*-butanol and 3% acetic acid). Column chromatography was carried out on Sorbsil C60, 40-60 mesh silica. Reverse phase analytical HPLC (RP HPLC) was performed using a Hewlett Packard HP1100 Chemstation, and compounds detected by ELS detector. All solvents used were HPLC grade. Microwave assisted heating was carried out by irradiating the mixture in a Smith Synthesiser at 2.45 GHz (available from Personal Chemistry). All reactions were carried out at room temperature unless otherwise stated.

### 1.2. Synthesis of resin bound scaffold 5

Resin bound scaffold **5** was prepared as previously described in Lebreton *et al*, *Tetrahedron*, 2003, **59**, 3945.

### 1.3. Synthesis of isocyanate $AB_3$ monomer 6

Isocyanate monomer **6** was synthesized as previously described in Fromont *et al*, *Chem. Commun.* 2000, 283.

## 1.4. General procedures for the synthesis of the dendrimers

- **Solid phase synthesis of Gen n.5 dendrimers (n = 0, 1, or 2)**

Isocyanate monomer **6** (1.2 eq), DMAP (0.1 eq) and DIPEA (2 eq) were dissolved in DCM/DMF (1/1). The resulting solutions were added to the respective resin bound scaffold **5** or dendrimers Gen n.0 (1.0 eq, with reference to the theoretical number of free amino groups on the resins in mmol) which were pre-swollen in DCM for 20 min. The mixtures were shaken for 1 to 3 days or irradiated with microwaves at 100 $^{o}$C for 60 min and completion of reactions was monitored by a qualitative FT-IR (presence of carbonyl ester at 1730 $cm^{-1}$). The resins were filtered and washed thoroughly with DMF, DMF/THF (1/1), THF, MeOH, DCM, $Et_2O$ (3 x 3 mL each) and dried *in vacuo*.

- **Solid phase synthesis of Gen n.0 (n = 1, 2, or 3) dendrimers**

Propane-1,3-diamine in methanol was added to the respective dendrimers Gen n.5 and the mixtures were shaken for 3 days. The progress of reactions was monitored by FT-IR (disappearance of carbonyl ester at 1730 $cm^{-1}$). The resins were filtered and washed thoroughly with DMF, DMF/THF (1/1), THF, MeOH, DCM, $Et_2O$ (3 x 3 mL each) and dried *in vacuo***.**

- **Lysine Conjugation on resin bound dendrimer 7**

Lysine conjugation was achieved by coupling Fmoc-protected L-lysine to resin bound dendrimer 7 using HOBt/DIC (3 eq, with reference to the theoretical number of free amino groups on the resins in mmol) in DCM/DMF (1/1, 2 mL) and shaken overnight. Coupling of lysine (10 eq) could not be forced to completion after 8 cycles of coupling (this included 2x microwave heating at 120 $^{o}$C for 60 min). The resin was filtered and washed with DCM (2 x 3 mL), DMF (2 x3 mL), DCM (2 x 3 mL), MeOH (2 x 3 mL) and $Et_2O$ (2 x 3 mL). The resultant resins were treated with 20% piperidine in DMF (2 mL) with two cycles of 30 min and dried *in vacuo* after washing (as above) to yield the resin bound deprotected dendrimers.

- **Acidolytic liberation of dendrimers from the solid support**

The respective resins bound dendrimers were swollen in DCM for 20 min. TFA/DCM/$H_2O$ (9.5/0.25/0.25) was added and the mixtures were shaken for 3 h. The solutions were filtered and the

resins were washed with DCM and dried *in vacuo* to yield the desired products **8** and **9** as TFA salts. The yields of the products were calculated based on the molecular weight of fully protonated TFA salts relative to initial loading of aminomethyl resin (0.61 mmol/g).

## 1.5. Characterization of dendrimers G 0.5-2.5

### Dendrimer G 0.5

Cleavage of resin G 0.5 (50 mg) afforded a colourless oil (20 mg, 62%). $^{1}$H-NMR (400 MHz, $CD_3CN$): δ 3.65 (t, *J* = 6 Hz, 12H), 3.64 (s, 18H), 3.56 (s, 12H), 3.23 (t, *J* = 6 Hz, 4H), 2.91 (br s, 4H), 2.52 (t, *J* = 6 Hz, 12H), 1.79 (m, 4H); $^{13}$C-NMR (100 MHz, $CD_3CN$): δ 171.8, 159.5, 69.3 ($CH_2$), 66.5 ($CH_2$), 58.9, 50.9 ($CH_3$), 43.8 ($CH_2$), 34.9 ($CH_2$), 34.2 ($CH_2$), 26.8 ($CH_2$). IR (neat) ν 3381, 1737, 1653, 1200 cm$^{-1}$. MS (ES+): m/z 471.8 [$(M+2H)^{2+}$, 65%], 942.4 [$(M+H)^{+}$, 100%].

### Dendrimer G 1.0

Cleavage of resin G 1.0 (48.0 mg) afforded a colourless oil (49.4 mg, 85%). $^{1}$H-NMR (400 MHz, $D_2O$): δ 3.42 (t, *J* = 6 Hz, 12H), 3.36 (s, 12H), 3.01 (t, *J* = 6 Hz, 12H), 2.86 (t, *J* = 6 Hz, 4H), 2.82-2.71 (m, 16H), 2.22 (t, *J* = 6 Hz, 12H), 1.63-1.53 (m, 16H); $^{13}$C-NMR (100 MHz, $D_2O$): δ 174.7, 161.6, 69.7 ($CH_2$), 67.7 ($CH_2$), 59.3, 45.4 ($CH_2$), 37.2 ($CH_2$), 36.5 ($CH_2$), 36.3 ($CH_2$), 36.2 ($CH_2$), 26.9 ($CH_2$), 26.7 ($CH_2$). IR (neat): ν 3317, 1640 cm$^{-1}$. MALDI-TOF: calc. for $C_{52}H_{108}N_{17}O_{14}$ $(M+H)^{+}$: 1194.8, found: 1194.6.

### Dendrimer G 1.5

Cleavage of resin G 1.5 (26.0 mg) afforded a colourless oil (16.7 mg, 28%).; $^{1}$H-NMR (400 MHz, $CD_3CN$): δ 3.70-3.65 (m, 102H), 3.59 (s, 48H), 3.30-3.20 (m, 16H), 3.11 (t, *J* = 6 Hz, 12H), 3.00 (br s, 4H), 2.54 (t, *J* = 6 Hz, 36H), 2.41 (br s 12H), 1.84 (br s, 4H), 1.61 (q, *J* = 6 Hz , 12H); $^{13}$C-NMR (100 MHz, $CD_3CN$): δ 171.7, 158.0, 69.4 ($CH_2$), 67.2 ($CH_2$), 66.5 ($CH_2$), 58.7, 50.9 ($CH_3$), 45.0 ($CH_2$), 36.8 ($CH_2$), 36.2 ($CH_2$), 34.1 ($CH_2$), 29.7 ($CH_2$), 28.0 ($CH_2$). IR (neat): ν 3350, 1735, 1640 cm$^{-1}$.

**Dendrimer G 2.0**

Cleavage of resin G 2.0 (20.4 mg) afforded a colourless oil (19.3 mg, 24%). $^{1}$H-NMR (400 MHz, $D_2O$): δ 3.64 (t, *J* = 6.0 Hz, 48H), 3.54 (s, 48H), 3.22 (t, *J* = 7 Hz, 36H), 3.12 (t, *J* = 7 Hz, 12H), 3.08 (br s, 4H), 3.01 (t, *J* = 7 Hz, 16H), 2.96-2.90 (m, 36H), 2.45-2.40 (m, 48H), 1.80 (q, *J* = 7 Hz, 40H), 1.57 (q, *J* = 7 Hz, 12H). $^{13}$C-NMR (100 MHz, $D_2O$): δ 174.6, 174.1, 159.7, 69.7 ($CH_2$), 67.7 ($CH_2$), 59.2, 45.6 ($CH_2$), 37.2 ($CH_2$), 36.5 ($CH_2$), 36.3 ($CH_2$), 29.3 ($CH_2$), 26.9 ($CH_2$). IR (neat): ν 3299, 1638 cm$^{-1}$. MALDI-TOF: calc. for $C_{190}H_{378}N_{59}O_{56}$ $(M+H)^+$: 4384.4, found: 4385.5.

**Dendrimer G 2.5**

Cleavage of resin G 2.5 (45.0 mg) afforded a colourless oil (20.5 mg, 6%). $^{1}$H-NMR (400 MHz, $CD_3CN$): δ 3.70-3.65 (m, 318H), 3.60 (s, 156H), 3.30-3.20 (m, 52H), 3.15-3.05 (m, 52H), 2.54 (t, *J* = 6 Hz, 108H), 2.41 (br s 48H), 1.84 (br s, 4H), 1.65-1.55 (m, 48H). $^{13}$C-NMR (100 MHz, $CD_3CN$): δ 171.7, 171.3, 157.9, 69.4 ($CH_2$), 67.3 ($CH_2$), 66.5 ($CH_2$), 58.6, 50.9 ($CH_2$), 36.7 ($CH_2$), 36.2 ($CH_2$), 34.2 ($CH_2$), 29.9 ($CH_2$). IR (neat): ν 3321, 1732, 1651 cm$^{-1}$.

## 1.6. Characterization of dendrimers 8 and 9

### Dendrimer 8

Cleavage of resin **7** (30.0 mg) afforded **8** as a colourless oil (15.0 mg, 4%). $^{1}$H-NMR (400 MHz, $D_2O$): δ 3.64 (t, *J* = 6 Hz, 156H), 3.45 (s, 156H), 3.15 (t, *J* = 7 Hz, 108H), 3.05 (t, *J* = 7 Hz, 48H), 2.90 (t, *J* = 7 Hz, 48H), 2.85 (t, *J* = 7 Hz, 108H), 2.37-2.32 (m, 156H), 1.95 (q, *J* = 7 Hz, 108H), 1.50 (q, *J* = 7 Hz, 48H); $^{13}$C-NMR (100 MHz, $D_2O$): δ 174.8, 159.7, 69.9 ($CH_2$), 67.9 ($CH_2$), 59.4, 37.4 ($CH_2$), 36.5 ($CH_2$), 29.5 ($CH_2$), 27.0 ($CH_2$). IR (neat) ν 3287, 1636 cm$^{-1}$.

### Dendrimer 9

Cleavage of resin bound polylysine dendrimer (29.5 mg) afforded compound **9** as a colourless oil (19.0 mg, 3%). $^{1}$H-NMR (400 MHz, $CD_3OD$): δ 3.90 (br s, 50H), 3.75-3.65 (m, 312H), 3.35-3.20 (m, 216H), 3.13 (br s, 96H), 3.08-2.95 (m, 100H), 2.48 (br t, *J* = 7 Hz, 156H), 2.00-1.90 (m, 100H), 1.82-1.65 (m, 156H), 1.60-1.45 (m, 100H). $^{13}$C-NMR (100 MHz, $CD_3OD$): δ 174.0, 170.4, 71.1 ($CH_2$), 69.6 ($CH_2$), 60.5, 54.2 (CH), 40.2 ($CH_2$), 38.0 ($CH_2$), 37.6 ($CH_2$), 32.0 ($CH_2$), 30.0 ($CH_2$), 28.0 ($CH_2$), 22.9 ($CH_2$). IR (neat) ν 3331, 1670.

Integration analysis of the α C***H*** (3.90, br s, 50H) from the L-lysine residues relative to the integration of the -COC***H***$_2$CH$_2$O- (2.48, br t, J = 7 Hz, 156H) from all the branches of the dendrimer was used to determined that dendrimer 9 contains (within NMR experimental error) approx 50 L-lysine residues.

## 2. DNA binding studies

DNA binding affinities were studied at two DNA/sample ratios (w/w), 1:10 and 1:20, using agarose gel electrophoresis. DNA/sample complexes at a ratio of 1:10 were made by transferring the sample (2 μL, 1.0 μg/μL) into an Eppendorf tube. Each sample was further diluted with TE buffer (3.6 μL). Plasmid DNA (pEGFP-N1, 0.4 μL, 0.5 μg/μL) was added to each sample to make a total volume of 6 μL and the solutions were successively mixed. The DNA/sample complexes at 1:20 (w/w) were prepared as above except 4 μL of sample and 1.6 μL of TE buffer were used. The DNA complexes were incubated at room temperature for 30 min. Bromophenol blue-gel-loading buffer (4 μL, 0.25% bromophenol blue and 40% w/v sucrose in water) was added to each complex sample, mixed, and each sample (10 μL) was loaded onto a 1 % agarose gel (1x TAE buffer). The gel was run at constant voltage (100 V) for 1 h, and DNA bands were visualized under UV light with ethidium bromide staining.

## 3. Biological Part

### 3.1. Cell lines and cell culture

Human embryonic kidney cells (HEK293T), human cervix adenocarcinoma cells (HeLa) and mouse melanoma cells (B16F10) were provided by Professor Tim Elliot (Cancer Science Division, School of Medicine, Southampton University). Mouse neuroblastoma and rat neurone hybrid cells (ND7) were provided by Dr. Lars Sundstrom (Clinical Neurosciences Division, School of Medicine, Southampton University). The cell lines (HEK293T, B16F10 and ND7 cells) were grown at 37 $^{o}$C under 5% $CO_2$ in a complete medium: Dulbecco's modified Eagle medium (DMEM, 4.5 mg/mL glucose) supplemented with 10% heat inactivated fetal bovine serum (FBS), 4 mM L-glutamine and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin). HeLa cells were maintained under the same conditions except DMEM was replaced by Roswell Park Memorial Institute (RPMI) 1640 media. All cell culture reagents were obtained from Invitrogen Ltd. (Inchinnan Busines Park, Paisley).

### 3.2. Formation of dendrimer/DNA complexes

The formation of dendrimer/DNA complexes is controlled by electrostatic interactions between the DNA and the protonated amino groups of the dendrimers in which charge neutralization of both components or net positive charge of the complexes results in changes in the properties of the complexes as described earlier. In this study, the fraction of amino groups that were protonated at pH 7.4 was unknown; therefore a weight ratio of dendrimer/DNA (w/w) instead of a charge ratio was applied when the dendriplexes were formed (see Table 1 for the theoretical N/P ratio that would correspond to each weight ratio used).

| Compound | DNA used (μg) | Dendrimer used (μg) | Dendrimer/DNA w/w ratio | Dendrimer/DNA N/P ratio |
|---|---|---|---|---|
| 8 | 0.3 | 3 | 10 : 1 | 9 : 1 |
| 8 | 0.3 | 6 | 20 : 1 | 18 : 1 |
| 9 | 0.3 | 3 | 10 : 1 | 10.4 : 1 |
| 9 | 0.3 | 6 | 20 : 1 | 20.8 : 1 |

**Table 1.** Charge and weight ratio correspondence.

*Transfection protocol:* complexes were formed by mixing the corresponding amount of dendrimer 8 or 9 (3 or 6 μL, 1 μg/μL) with the plasmid DNA (1 μL, 0.3 μg/μL) and then diluted in serum-free media to 20 μL. The mixture was vortexed for 10 s and incubated at room temperature for 30 min before adding the cells. The commercially available transfection reagents, Superfect and Effectene, were mixed with DNA at the ratios recommended by the manufacturer. All experiments were carried out with cells incubated in serum-containing media.

### 3.3. Transfection procedure.

Cells (HEK293T, HeLa, ND7 and B16F10 cells) were grown as described before up to 80% confluence. Subsequently, cells were detached with trypsin/EDTA, re-suspended in media and counted. $2x10^4$ cells were platted per well of a 96-well plate and incubated at 37 °C under 5% $CO_2$ overnight. Prior to adding complexes, the cell culture medium was removed and replaced with fresh cell growth medium (80 μl, containing serum and antibiotics).

Transfection experiments were accomplished by transfecting 0.3 μg of pEGFP-N1 per well. Complexes (20 μL) were prepared as explained before and then transferred into the wells of the 96-well plate. Cells were exposed to the complexes for 4 h at 37 °C under 5% $CO_2$ (each assay being performed in triplicate). The media containing the DNA/dendrimer complex was then removed and the cells were incubated with fresh growth medium (100 μL) for 48 h. Transfected cells were directly visualized using a Leica fluorescence microscope and then analysed by flow cytometry. Transfection efficiency was calculated after subtracting the value of untransfected cells.

Superfect and Effectene were used as positive controls and complexes were prepared according to the procedure recommended by the manufacturer (Qiagen).

Naked DNA, DNA-free reagents and complex-free cells served as negative controls.

### 3.4. Flow cytometry analysis of transfected cells

Cells were grown in complete media as mentioned above. After treatment with the respective agents for the required times, cells were washed with PBS (2 x 200 μL), harvested with trypsin/EDTA (50 μL) and re-suspended in PBS (150 μL) containing 1% FBS. The transfection efficiency was determined by flow cytometry analysis using a FACSAria flow cytometer (Becton Dickinson)

equipped with an argon laser emission at 488 nm. Fluorescein isothiocyanate (FITC, 530/30nm) band pass filters was used for the study of transfection efficiency and the data were analysed using the FACSDiva software (Becton Dickinson).

In order to analyze the untransfected cell controls, histograms of flow cytometry data were generated as a function of cell number and fluorescence intensity (**Figure 2**). From the corresponding analysis for each cell line, it was selected a setting limit of fluorescence intensity representing the untransfected cell population. The population of cells with fluorescence intensity below this setting limit in each cell control was ≥ 98%.

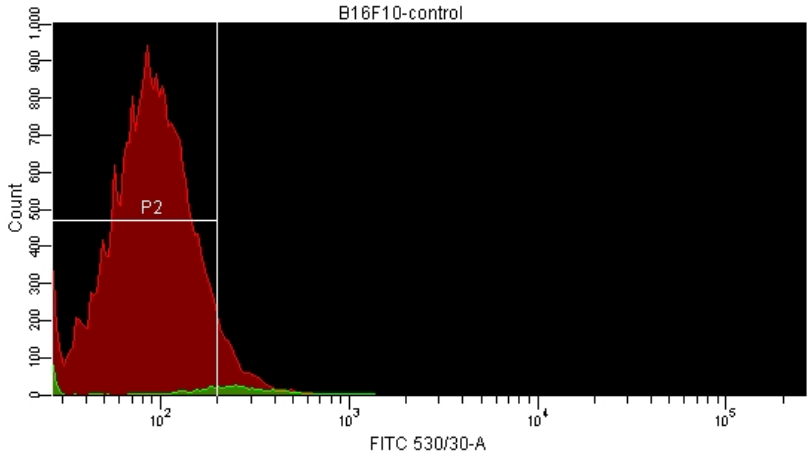
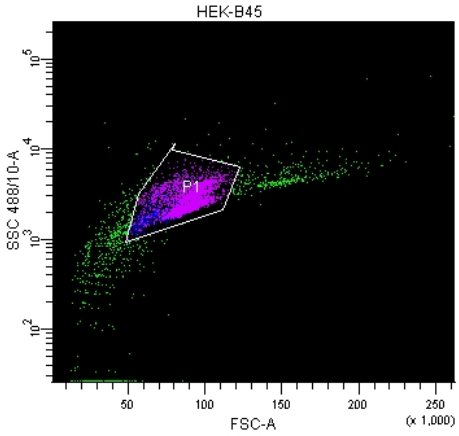



**Figure 2** Histogram of B16F10 cell control as analysed by flow cytometry.

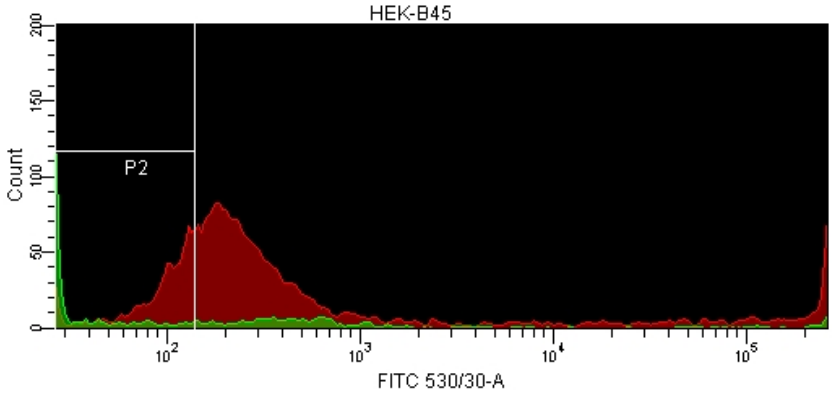
For each analysis, cells were first plotted as a function of their physical parameters (forward and side light scatter, FSC and SSC, respectively), FSC indicates relative differences in the size of the cells whereas SSC indicates relative differences in the internal complexity or granularity of the cells (**Figure 3a**), and cells were gated to exclude dead cells and cell debris. Histograms of flow cytometry data were generated as a function of cell number and fluorescence intensity (**Figure 3b**).

Transfection efficiencies were reported as a percentage of cells expressing fluorescence after subtracting the control cells (**Figure 3c**) based on the statistical data previously generated from the analysis of the corresponding cell controls.

A total of 5000 or 10000 events per sample were analyzed.




(a)




(b)

| | | | |
|---|---|---|---|
| Experiment Name: | tranfection HEK 20-08-04 plate B | | |
| Specimen Name: | HEK | | |
| Tube Name: | B45 | | |
| Record Date: | Aug 20, 2004 5:33:25 PM | | |
| $OP: | Administrator | | |
| | | | FITC 530/30-A |
| Population | #Events | %Parent | Mean |
| All Events | 5,000 | #### | 24,723 |
| P1 | 4,068 | 81.4 | 26,564 |
| P2 | 872 | 21.4 | 93 |
| NOT(P2) | 3,196 | 78.6 | 33,787 |
| NOT(P1) | 932 | 18.6 | 16,687 |

(c)

**Figure 3** Quantification of HEK293T cells expressing GFP after treatment with DNA/dendrimer **9** complexes (stated as B45 in the above figures): (a) Contour plot of flow cytometry data as a function of SSC and FSC. P1 indicates the gated cells; (b) Histogram of flow cytometry data as a function of cell number and fluorescence intensity. P2 indicates the number of cells excluded from the setting limit of the fluorescence intensity; (c) Statistical data was generated using the FACSDiva software. (Dose of pEGFP-N1: 0.3 μg, WR: 10:1, exposure time: 24 h, confluency of cells: 80%, total incubation time: 48 h.)

As shown in Figure 4, dendrimer **9** achieved 83% transfection efficiency in B16F10, with resulting in two populations of cell (one with high GFP expression and another with lower GFP expression).

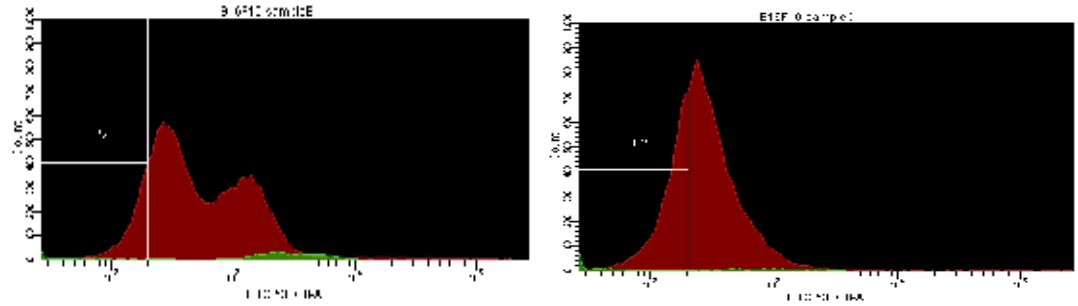


**Figure 4** Histogram of B16F10 cells expressing GFP after treatment with dendrimer **9** (left, 83% transfection efficiency) and Superfect (right, 68% transfection efficiency) as analysed by flow cytometry. The scale of all the histograms (plotted as a function of cell number against fluorescence intensity, x-axis) are identical for all histograms. Thus the histogram shows a shift to the right (indicating an increase in fluorescence) compared to the control when transfected.

### 3.5. MTT assay

The cells were seeded for 24 h (or 80% confluence) prior to treatment in a 96-well plate at $2x10^4$ cells per well (in 200 µL of complete medium). Cells were incubated at 37 $^oC$ under 5% $CO_2$. Appropriate concentrations of test reagents were added to the wells and the mixtures were incubated for 48 h. After incubation, the reagents were removed and replaced with phenol free complete media (90 µL), 10 µL of MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, 5 mg/mL, dissolved in medium without phenol red and serum] was added to each well. Following 3 h incubation, the resulting formazan crystals were dissolved by adding solubilisation solution (100 µL, 10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol). Absorbance was measured at 570 nm using a Bio-Rad Benchmark microplate reader and converted to percentage of cell viability relative to control cells.