

# **Multifunctionalised cationic fullerene adducts for gene transfer: design, synthesis and DNA complexation**

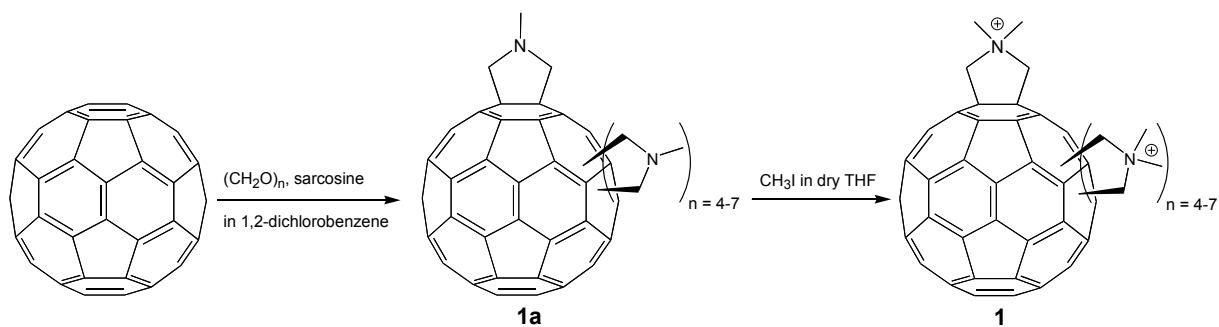
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Kostarelos,<sup>\*c</sup> Maurizio Prato<sup>\*b</sup> and Alberto Bianco<sup>\*a</sup>**

## **Supporting Information**

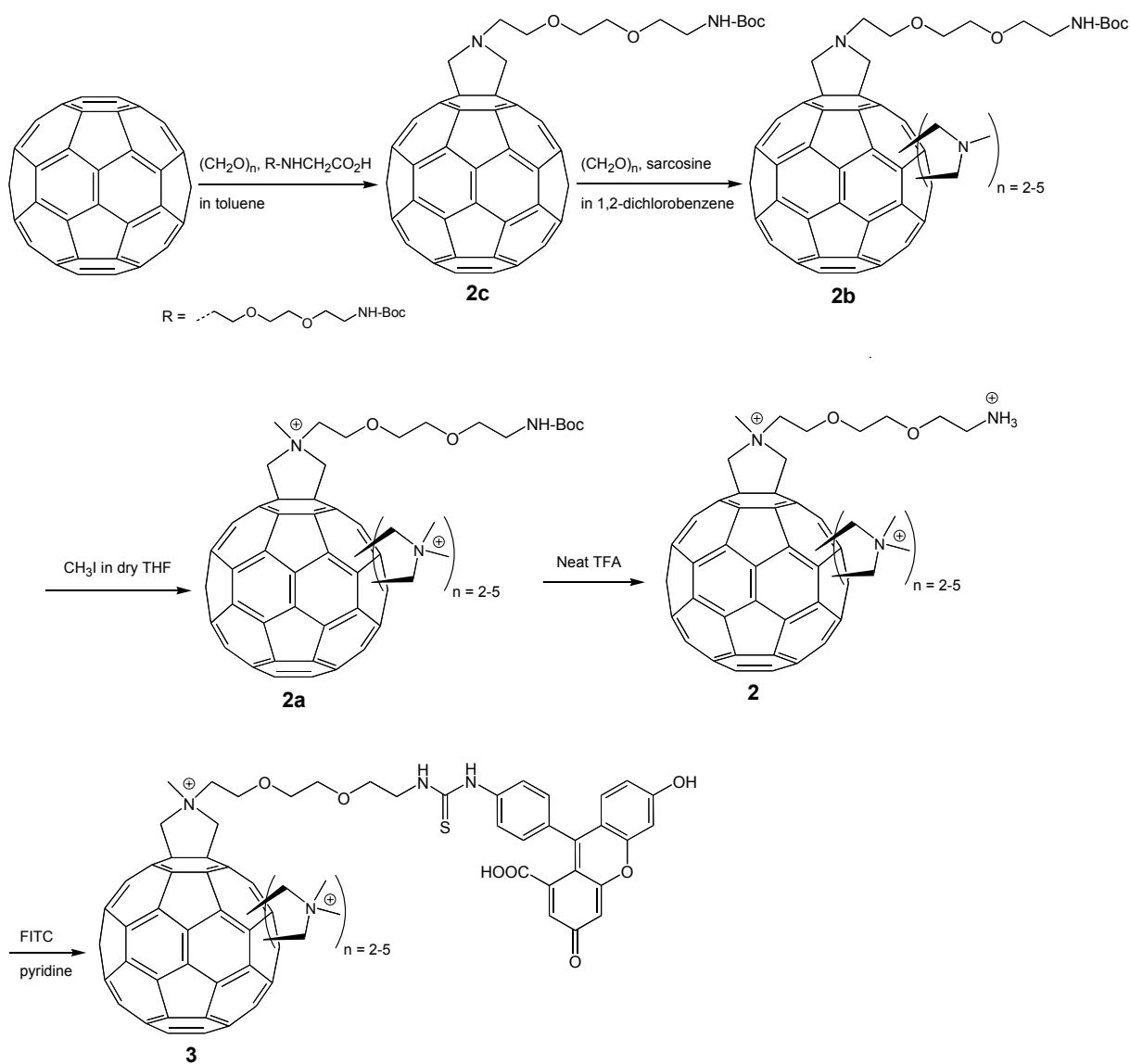
### **Experimental Section**

**General.** All reagents and solvents were obtained from commercial suppliers and used without further purification. C<sub>60</sub> was purchased from Bucky-USA (Houston, TX). The reactions were monitored by thin-layer chromatography (TLC) on silica gel (F<sub>254</sub> Merck) and the products were visualised on aqueous potassium permanganate or ninhydrine spray followed by heating. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded in CDCl<sub>3</sub>, D<sub>2</sub>O and DMSO-*d*<sub>6</sub> using a Bruker DPX 300 spectrometer equipped with a 5 mm <sup>1</sup>H/<sup>13</sup>C probe for both proton (300 MHz) and carbon (75 MHz). The peak values are given as ppm (δ), using the tetramethylsilane or the residual deuterated solvent protons as a reference. DOSY spectra were recorded on a Bruker Avance500 spectrometer, at 11.7 tesla, at the resonating frequency of 500.13 MHz for <sup>1</sup>H, using a BBI Bruker 5 mm gradient probe. The temperature was regulated at 298 K and no spinning was applied to the NMR tube. The diffusion NMR experiments were performed with a Pulsed-Field Gradient STimulated Echo (PFGSTE) sequence, using bipolar gradients.<sup>1,2</sup> Chromatographic purifications were done with silica gel Merck (Kieselgel 60, 40-60 μm, 230-400 mesh ASTM) in standard column. Organic phases were dried with sodium sulphate. RP-HPLC was performed on a Macherey-Nagel C<sub>18</sub> column using a linear gradient of A: 0.1% TFA in water and B: 0.08% TFA in acetonitrile, 0-55%B in 20 min at 1.2 mL/min flow rate. A detailed characterisation and identification of the polycationic fullerene derivatives by MALDI-tof or electrospray mass spectrometry was impossible as an important amount of peaks corresponding to multi-charged species and to fragmentations of the compounds was observed.

## Synthesis of the multifunctionalised cationic fullerene adducts

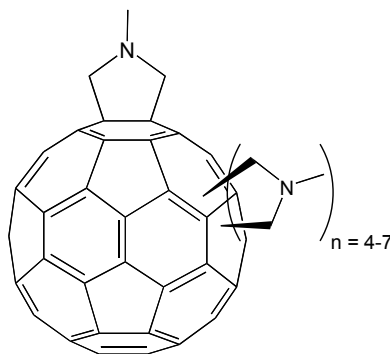


Scheme S1



Scheme S2

### Synthesis of poly-*N*-methylfulleropyrrolidine derivative 1a



To a solution of  $C_{60}$  (100 mg, 0.14 mmol) in 1,2-dichlorobenzene (100 mL) *para*formaldehyde (210 mg, 7 mmol) and sarcosine (620 mg, 7 mmol) were added (Scheme S1). The reaction was stirred at 180°C during 1 h. Once the reaction was at room temperature, the solution was directly poured on a flash column chromatography and the mixture of fullerene adducts was recovered using DCM followed by a solution of DCM/ $Et_3N$ /MeOH 94/3/3. The organic solvents were evaporated to obtain a dark red solid. Amount: 125 mg.  $^1H$  NMR (500 MHz,  $CDCl_3$ ):  $\delta$  (ppm) 3.98-2.87 (br, m, 4H,  $CH_2$ ) 2.75-2.22 (br, m, 3H,  $CH_3$ ). The molecular formulas of the main components identified by mass spectrometry were:  $C_{75}H_{35}N_5$ ,  $C_{78}H_{42}N_6$ ,  $C_{81}H_{49}N_7$ ,  $C_{84}H_{56}N_8$  (M.W. 1005.29, 1062.25, 1119.40, 1176.46). MALDI-tof  $m/z$ : 1006.04 (20%), 1063.08 (30%), 1120.14 (35%), 1177.20 (10%)  $[M+H]^+$ .

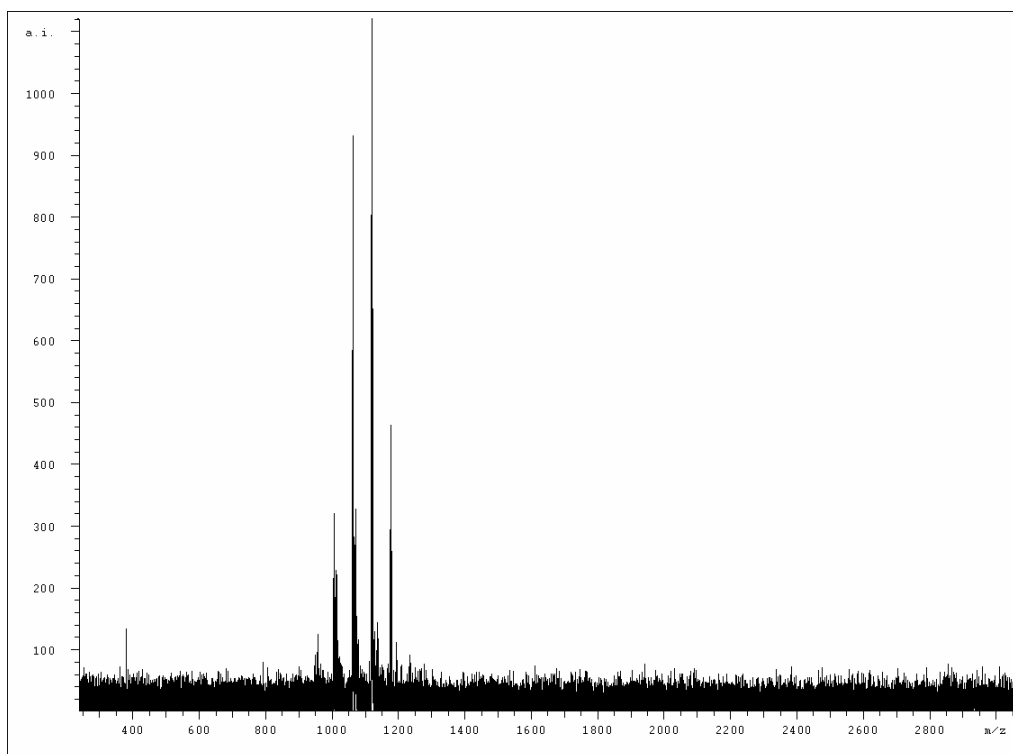
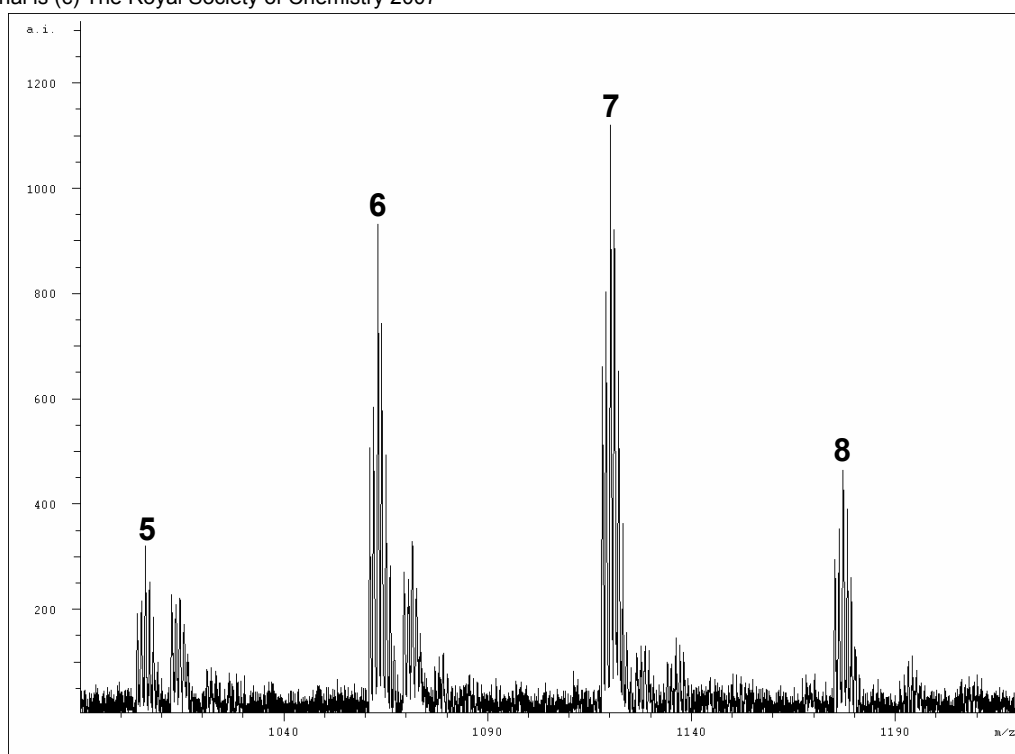
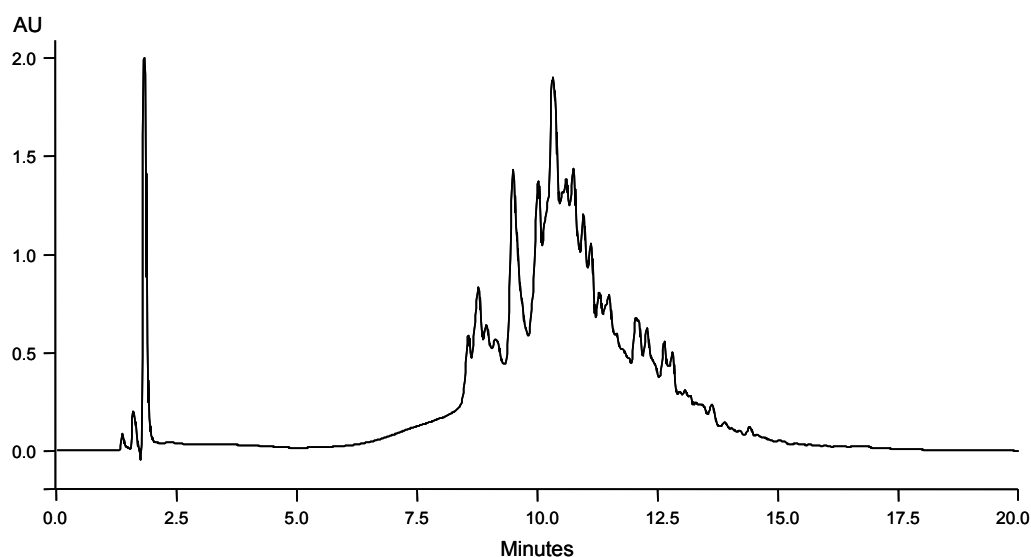


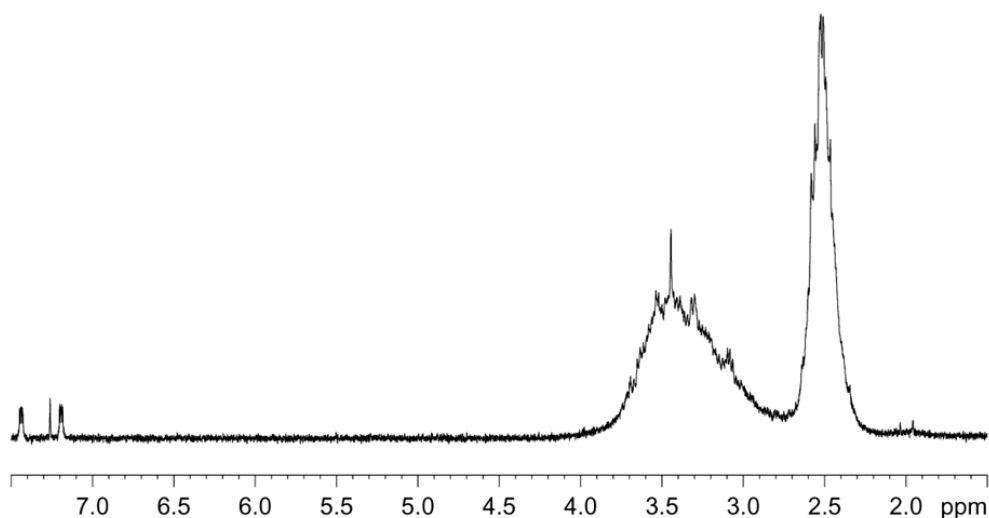
Figure S1. MALDI-tof spectrum of the fullerene derivative 1a.



**Figure S2.** Zoom of the MALDI-tof spectrum of the fullerene derivative **1a** displaying the 4 main adducts.

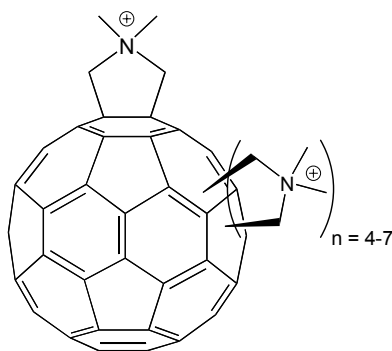


**Figure S3.** RP-HPLC chromatogram of fullerene derivative **1a** displaying a massif of peaks between 7 and 14 minutes attributed to the different regioisomers formed during the multiple 1,3-dipolar cycloaddition reaction. Gradient: 0-55%B in 20 min. A: 0.1% TFA in water; B: 0.08% TFA in acetonitrile. Flow rate: 1.2 ml/min. Detection: 214 nm.



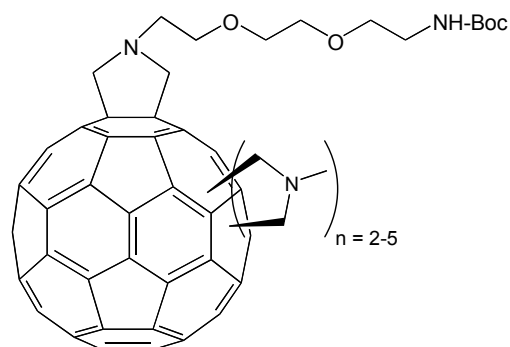
**Figure S4.** NMR spectrum of fullerene derivative **1a** in  $\text{CDCl}_3$ .

### Synthesis of poly-*N,N*-dimethylfulleropyrrolidinium derivative **1**



To a solution of **1a** (20 mg) in anhydrous THF (1 mL) iodomethane (1 mL, 16 mmol) was added and the reaction was stirred in a sealed tube at room temperature overnight. The mixture was centrifuged at 15000 g during 5 minutes. The precipitate was collected and washed 4 times using a mixture of MeOH/Et<sub>2</sub>O to afford 22 mg of an orange powder. <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O):  $\delta$  (ppm) 3.71-3.25 (br, m, CH<sub>2</sub>), 2.50-2.23 (br, m, CH<sub>3</sub>). Hydrodynamic radius calculated by DOSY in D<sub>2</sub>O: 9.81 Å. The average amount of positive charges per fullerene molecule is ~6.05.

### Synthesis of poly-*N*-methylfulleropyrrolidine derivative **2b**



To a solution of the fullerene monoadduct **2c** prepared as reported in the literature<sup>3</sup> (100 mg, 0.10 mmol) in 1,2-dichlorobenzene (100 mL) *para*formaldehyde (60 mg, 2.01 mmol) and sarcosine (180 mg, 2.01 mmol) were added (Scheme S2). The reaction was stirred at 120°C during 2 h. Once the reaction was at room temperature, the solution was directly poured on a flash column chromatography and the mixture of fullerene adducts recovered using DCM followed by a solution of DCM/Et<sub>3</sub>N/MeOH 98/1/1. The organic solvents were evaporated to obtain a dark red solid. Amount: 130 mg. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ (ppm) 4.07-2.93 (br, m, CH<sub>2</sub>) 2.79-2.39 (br, m, CH<sub>3</sub> pyrrolidine), 1.41 (br, s, CH<sub>3</sub> Boc). The molecular formulas of the main components identified by mass spectrometry were: C<sub>82</sub>H<sub>47</sub>N<sub>5</sub>O<sub>4</sub>, C<sub>85</sub>H<sub>54</sub>N<sub>6</sub>O<sub>4</sub>, C<sub>88</sub>H<sub>61</sub>N<sub>7</sub>O<sub>4</sub>, C<sub>91</sub>H<sub>68</sub>N<sub>8</sub>O<sub>4</sub> (M.W. 1165.36, 1222.42, 1279.48, 1336.54). MALDI-tof *m/z*: 1166.11 (5%), 1223.13 (25%), 1280.18 (45%), 1337.22 (20%) [M+H]<sup>+</sup>.

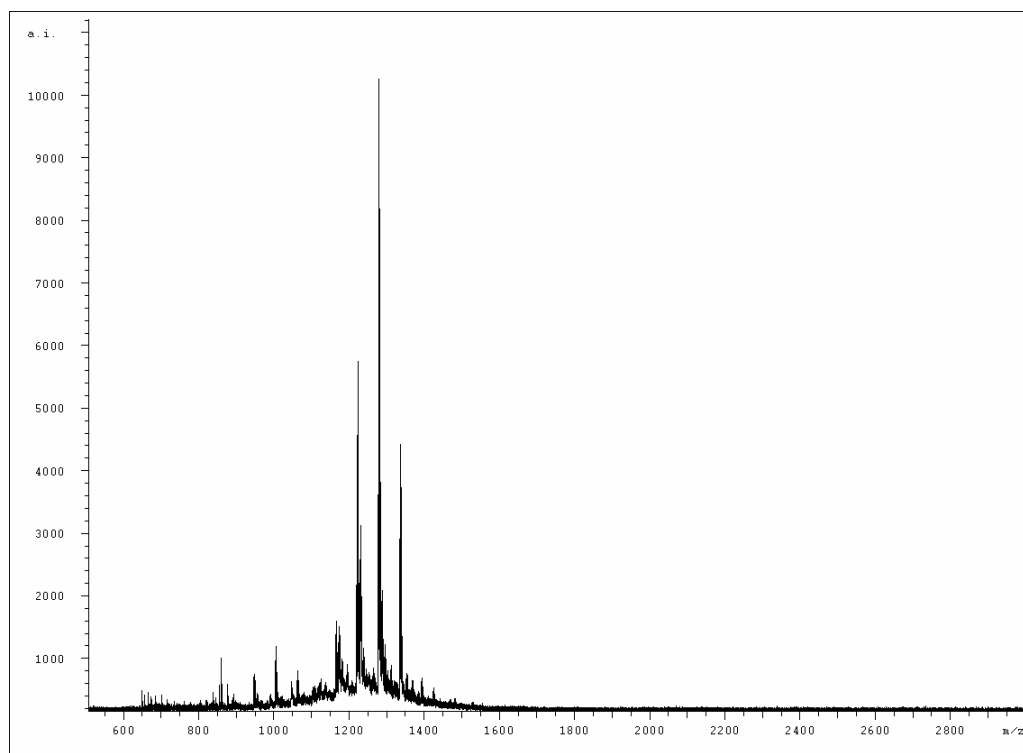
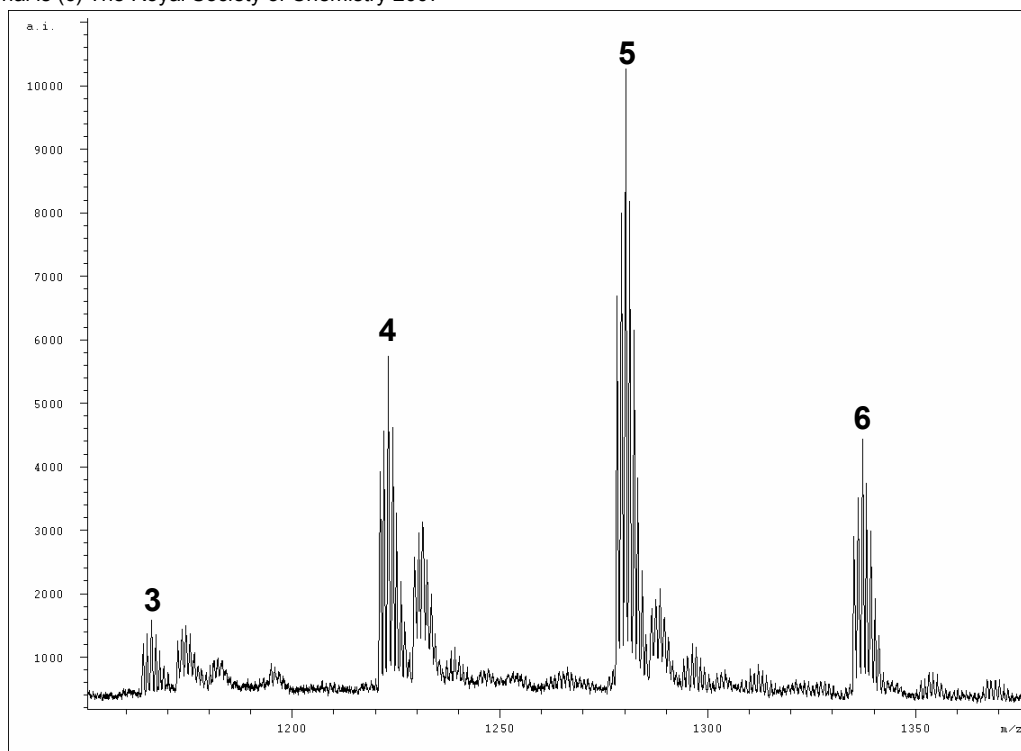
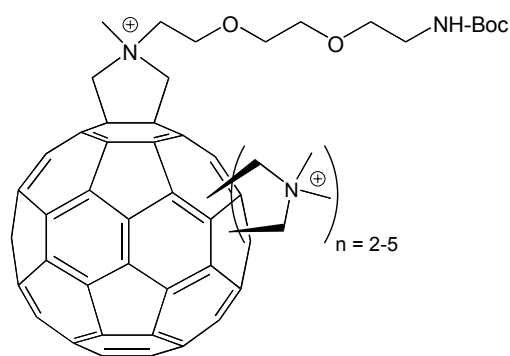


Figure S5. MALDI-tof spectrum of the fullerene derivative **2b**.



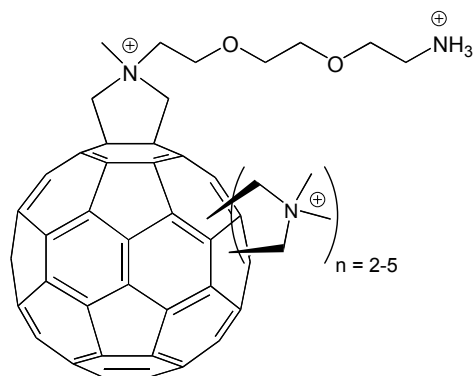
**Figure S6.** Zoom of the MALDI-tof spectrum of the fullerene derivative **2b** displaying the 4 main adducts.

### Synthesis of poly-*N*-dimethylfulleropyrrolidine derivative **2a**



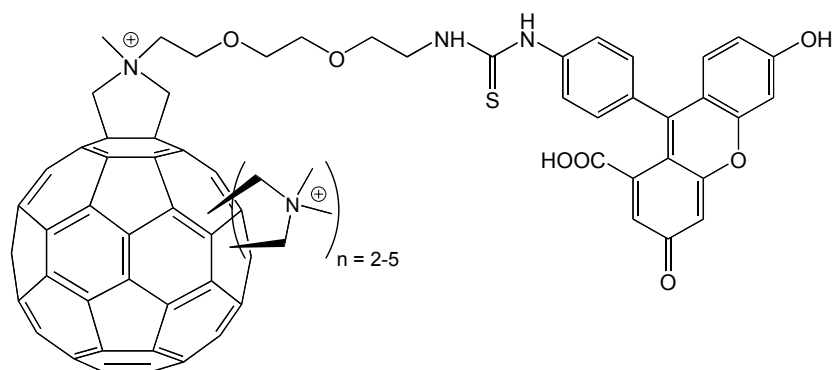
To a solution of **2b** (46 mg) in anhydrous THF (2 mL) was added iodomethane (500  $\mu$ L, 8 mmol) (Scheme S2). The reaction was stirred in a sealed tube for 12 h at room temperature. The mixture was centrifuged at 15000 g for 5 minutes. The product was reprecipitated 3 times in MeOH/Et<sub>2</sub>O to obtain a red solid. Amount: 53 mg. <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O):  $\delta$  (ppm) 3.87-3.45 (br, m, CH<sub>2</sub> and CH<sub>3</sub>), 1.39 (br, s, CH<sub>3</sub>). The average amount of positive charges per fullerene molecule is ~4.6.

## Synthesis of poly-*N*-dimethylfulleropyrrolidinium derivative 2



The fullerene derivative **2a** (53 mg) was stirred in TFA (5 mL) at room temperature during 40 minutes (Scheme S2). The acid was evaporated and the product reprecipitated 3 times by centrifugation at 15000 g for 5 minutes in MeOH/Et<sub>2</sub>O/hexane. Amount: 40 mg. <sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O):  $\delta$  (ppm) 3.92-3.25 (br, m, CH<sub>2</sub> and CH<sub>3</sub>). The average amount of positive charges per fullerene molecule is ~5.6.

## Synthesis of fluorescent poly-*N*-dimethylfulleropyrrolidinium derivative 3



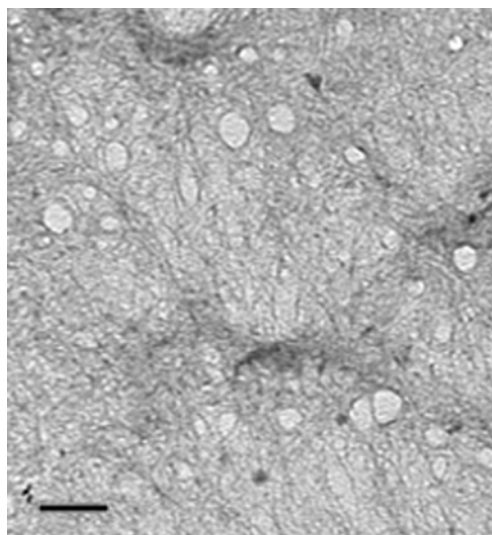
To a solution of **2** (50 mg) in pyridine (15 mL) fluorescein isothiocyanate (FITC) (18 mg, 0.046 mmol) was added. The reaction was stirred 12 hours at room temperature. The solvent was evaporated and the product was reprecipitated 10 times from MeOH to afford 40 mg of a dark brown solid. TLC and HPLC analytic control revealed no traces of free FITC. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm) 10.46-10.08 (m, aromatic protons), 8.86-7.72 (m, aromatic protons), 7.74-7.06 (m, aromatic protons), 6.65 (s, aromatic protons), 4.91-4.40 (m, CH<sub>2</sub>), 4.34-3.24 (m, CH<sub>2</sub> and CH<sub>3</sub>). Kaiser test: negative.



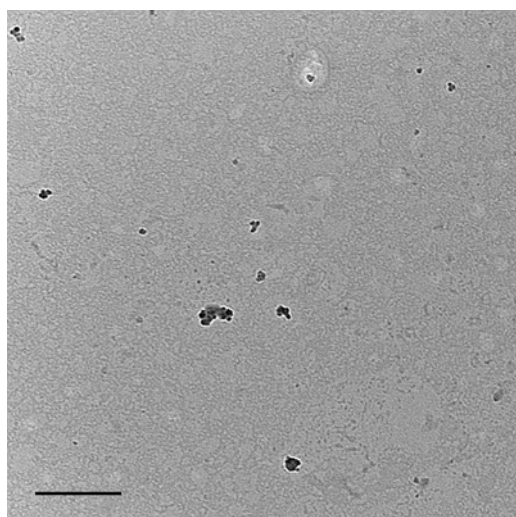
## Plasmid DNA/polycationic fullerene complexation studies

### Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on a Hitachi 600 HS microscope working at different accelerating voltage and at different magnification. Pictures were taken using a CCD high-resolution camera AMT. The samples were prepared on copper grid 200 meshes coated with carbon-Formvar from Electron Microscopy Sciences.



**Figure S7.** TEM image of pDNA solubilised in water and deposited on the grid. The solution was slowly evaporated before the observation. Scale bar corresponds to 500 nm.



**Figure S8.** TEM image of poly-*N*-dimethylfulleropyrrolidinium derivative **1** solubilised in water and deposited on the grid. The solution was slowly evaporated before the observation. Scale bar corresponds to 500 nm.

### **Gel electrophoresis**

Amounts of 0.2  $\mu\text{g}$  of pDNA (pCMV- $\beta\text{gal}$ , BD-Clontech, UK) diluted in deionised water or in serum-free DMEM cell culture media were complexed with the fullerene derivative **1** at different charge ratios. The complexes were allowed to form for 30 min at room temperature. To evaluate the degree of pDNA complexation with the fullerene derivatives, the complexes were loaded in a 2% agarose gel containing ethidium bromide. The gel was run for 5 h at 80 V in TAE buffer. Imaging of the gel was carried out with a UV light in the bio imaging system Gene Genius (Syngene, UK).

### **Surface plasmon resonance**

The BIAcore 3000 system, sensor chip CM5, surfactant P20, amine coupling kit containing NHS and EDC were from BIAcore (Uppsala, Sweden). All biosensor assays were performed in HBS running buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4). Immobilisation of fullerene derivative **2** was performed by injecting 35  $\mu\text{L}$  of **2** solution (100  $\mu\text{g}/\text{mL}$  in acetate buffer, pH 4.0) onto the surface of a sensor chip CM5 activated with EDC/NHS. This was followed by 20  $\mu\text{L}$  of ethanolamine hydrochloride, pH 8.5, to saturate the free active sites of the matrix. A 100 mM  $\text{H}_3\text{PO}_4$  solution was used to remove fullerene derivative non-covalently immobilised on the chip. All the binding experiments were carried out at 25°C with a constant flow rate of 20  $\mu\text{L}/\text{min}$ . pCMV $\beta$  pDNA (7.2 kbp) at concentrations from 2.62 nM to 21 nM was fluxed over the immobilised fullerene derivative **2** for 90 seconds and its dissociation was followed in the HBS buffer for 180 seconds. The immobilised fullerene derivative **2** was regenerated for 30 seconds with 100 mM  $\text{H}_3\text{PO}_4$ . The kinetic parameters were calculated using the BIAeval 4.1 software. Analysis was performed using the simple Langmuir binding model. The specific binding profiles were obtained after subtracting the response signal from the flow cell control and subtracting the response of the solvent. The fitting of the model was judged by the chi square value and randomness of residue distribution compared to the theoretical model.

### **Cell culture and treatment with fullerene derivative 3**

Cells were cultured in 25 or 75  $\text{cm}^2$  flasks (Corning, NY, USA). 6, 24 and 96 wells plates were purchased from Corning. Jurkat human T-lymphoma cell line was cultured in RPMI 1640 (Cambrex Bioscience) supplemented with gentamicin (10  $\mu\text{g}/\text{mL}$ ) and 10% of heat-inactivated fetal bovine serum. Cells were grown in suspension at 37 °C in a humidified atmosphere with 5%  $\text{CO}_2$ . Cell suspensions were prepared at a final concentration of  $5 \times 10^5$  cells/ml in 2 mL of medium containing fullerene derivative **3** at the final concentration

comprised between 0.1 and 10  $\mu\text{g/mL}$  (Stock solution: 0.1 or 1  $\text{mg/mL}$  of **3** in water), and incubated at 37 °C for 5 h. After incubation, the cells were washed twice with 2 mL of phosphate buffered saline (PBS) and then analysed for intracellular fullerene derivative **3** content by flow cytometry and fluorescence microscopy. Cell viability was evaluated by flow cytometry by assessing the change in cell size (FCS) and granularity (SSC).<sup>4</sup>

### Flow cytometry measurements

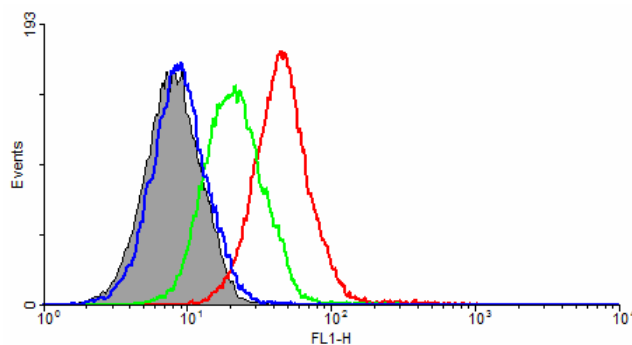
After two washes in PBS, cells were resuspended in 300  $\mu\text{L}$  of PBS and analysed with the flow cytometer FACSCalibur® operating at 488 nm excitation wavelength and detecting emission wavelengths with a 530/30 nm band-pass filter. At least 20,000 cells were acquired using the CellQuest 3.3 software (Becton & Dickinson) and cellular distribution of the FITC fluorescence was analysed with the WinMDI 2.9 freeware (Joseph Trotter, Scripps Research Institute).

### Confocal microscopy

After two washes in PBS, cells were resuspended in ready to use Fluorescent Mounting Medium (DakoCytomation) and mounted between glass slide and coverslip. The distribution of fluorescence was analysed using a Zeiss LSM 510 Meta confocal microscope operating at 488 nm excitation wavelength and detecting emission wavelengths with a 505-550 nm band-pass filter. Data were analysed using the ImageJ 1.37v freeware (Wayne Rasband, National Institutes of Health) and the LSM Reader 4.0d plugin (Patrick Pirrotte, Yannick Krempf and Jerome Mutterer, Institute for Molecular Biology of Plants, Strasbourg, France).

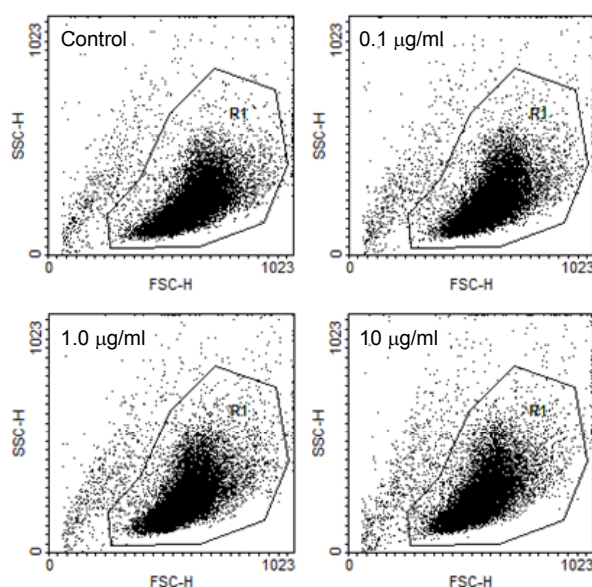
### Cell uptake study

The capacity of poly-*N*-dimethylfulleropyrrolidinium derivatives to penetrate into the cells was analysed to demonstrate the potential of this system for gene transfer. Preliminary experiments were performed on the fluorescent compound **3** using fluorescence-activated cell-sorting (FACS) analysis and confocal microscopy. Jurkat cells were incubated with different concentration of **3** to determine their global fluorescence and localisation inside the cell, respectively. FSC and SSC were recorded for the FITC-labelled fullerene derivative **3**. Jurkat cells were incubated with **3** at a concentration of 0.1, 1.0 and 10  $\mu\text{g/mL}$  during 5 h at 37 °C. As displayed in Figure S9, a clear dose-response uptake was observed.



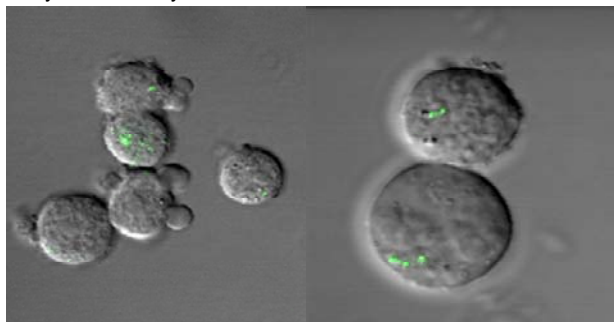
**Figure S9.** Dose-response of the internalisation after incubation of Jurkat cells for 5 h at 37 °C with increasing amount of fullerene derivative **3** (from 0.1 to 10  $\mu\text{g/ml}$ ). The grey surface represents the control cells. FL1-H corresponds to FITC intensity.

Flow cytometry allowed also to record information on the morphology of the cells. No changes on FSC and SSC parameters was observed using increasing amount of fullerene derivative **3** in comparison to the control (Fig. S10). From these observations, we can derive that the cationic poly-*N*-dimethylfulleropyrrolidine salts are non toxic up to 10  $\mu\text{g/mL}$ .



**Figure S10.** Flow cytometry assessment of the cell viability after 5 h, using increasing amount of fullerene derivative **3**. R1 comprises the region of living cells. FSC-H and SSC-H describe the morphology of the cells: FSC indicates the size and SSC the granularity of the cells. Cells comprised into R1 region have live cell morphology.

To obtain more details about the localisation of these compounds inside the cells, we used the confocal microscopy analysis. Jurkat cells were incubated for 5 h with compounds **3** at 10  $\mu\text{g/mL}$ . The images revealed that about 50% of the cells were FICT positive (Fig. S11). The florescence was manly distributed inside the cytoplasm.



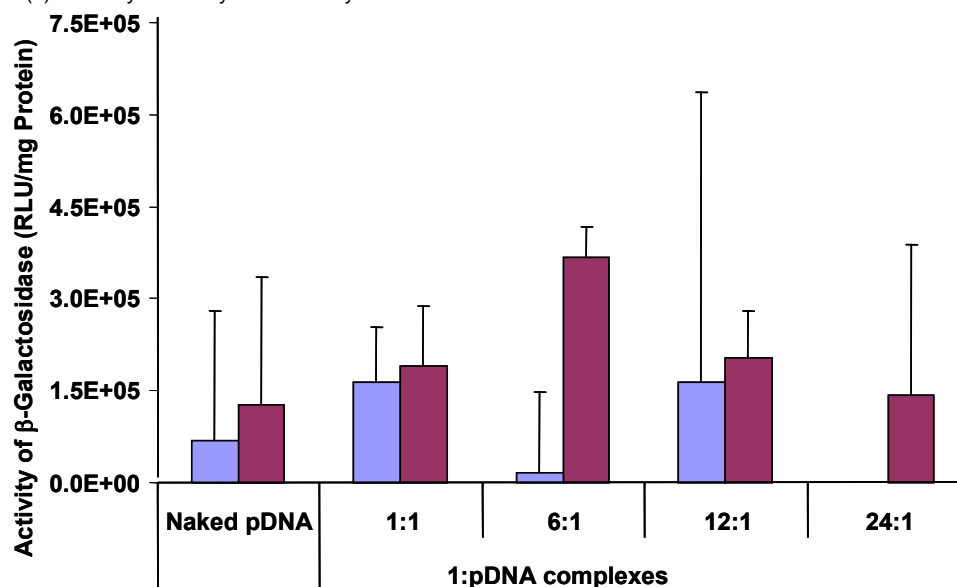
**Figure S11.** Confocal microscopy images of Jurkat cells incubated for 5 h at 37 °C with 10  $\mu\text{g/ml}$  of fullerene derivative **3**. Jurkat cells have an average diameter of 10  $\mu\text{m}$ .

The polycationic compound **3** is able to cross the cell membrane. Due to the positive charges, the molecule may be internalised by interacting with the negative charged cell membrane components. Further studies to elucidate the type of uptake mechanism are in due course.

### Gene transfer study

Human lung carcinoma cells (A549) were seeded in 24 wells plates with DMEM cell media supplemented with 10 % FBS and 1% penicillin/streptomycin. The cells were allowed to attach and form a monolayer overnight (confluence of 75%). Fullerene derivative **1**:pDNA complexes were formed by adding pDNA (diluted in water or in serum-free DMEM cell culture media; 200  $\mu\text{l}$ ) to the appropriate amount of fullerene derivative **1** (1 to 100  $\mu\text{g}$  in 200  $\mu\text{l}$  of deionised water) in order to achieve different positive:negative charge ratios. The two solutions were pipetted rapidly and then the complexes were allowed to form for 30 min at room temperature.

The complete media was removed and the cells were washed once with serum-free DMEM cell culture media. Aliquots of 100  $\mu\text{l}$  of each charge ratio complex (containing 0.5  $\mu\text{g}$  of pDNA) were added per well. The complexes were incubated with the cells in serum-free DMEM cell culture media (final volume of 500  $\mu\text{l}$ ) for 4 h at 37 °C, 5%  $\text{CO}_2$ . Afterwards, the transfection media was replaced by fresh and complete DMEM cell culture media and the cells were incubated for further 48 h (37 °C, 5%  $\text{CO}_2$ ). Finally, the cells were washed with PBS and lysed. The  $\beta$ -galactosidase expression was analysed using the Tropix Galactolight Plus Kit (Applied Biosystems, CA) and a Lumat LB9507 luminometer (Berthold Technologies, Germany). The content of protein per well was determined by BCA assay. Results are expressed as relative light units per mg of protein. All conditions were tested in triplicate.



**Figure S12.** Delivery and expression of pDNA by poly-*N*-dimethylfulleropyrrolidinium derivative **1**. Levels of marker gene ( $\beta$ -gal) expression in relative light units per mg of total protein in A549 cells. Different **1**:pDNA charge ratios were tested. Toxicity manifested as cell detachment and death was not observed throughout this study. Fullerene derivative **1** was solubilised in pure water (■) or in serum-free DMEM (■).

The levels of detected gene expression seem to be affected by the solution where the pDNA is diluted. The dilution of pDNA with serum-free DMEM cell culture media has consistently produced higher gene expression compared to naked DNA alone. However, the overall levels of gene expression were low compared to commercially available transfection agents. More work is needed to better understand the cellular uptake of the fullerene:DNA complexes in order to design adducts that would allow higher levels of gene expression.

## References

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