# A Peptide-Functionalized Dendronised Polymer for Selective Transfection in Human Liver Cancer Cells

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# 1 Experimental

## 1.1 Materials

The peptide SP94 (FITC-GCESFSIIHTPILPL-COOH) was synthesised by Synpeptide (Shanghai, China). Poly(amidoamine) 5.0G dendrimer (PAMAM) with an ethylenediamine core was purchased from Sigma Aldrich (#536709) as a 5 wt% solution in methanol and was dialysed against distilled water and lyophilised before being used *in vitro*. All other chemicals were purchased from Merck, Australia and used without further purification.

pEF1α-mCherry-sgRNA (5.3kb) was a gift from Prof Ryan Lister (Harry Perkins Institute of Medical Research and University of Western Australia). Briefly, mCherry was driven by the EF1α promoter and contained a short IVS sequence to enhance transcription of the fluorescent protein. The SV40 polyadenylation signal C-terminus of the IVS prevented transcriptional readthrough into the sgRNA expression cassette. The plasmid was amplified in *E. coli* (DH5α) and isolated using an endotoxin-free maxiprep kit (Qiagen).

## 1.2 Cell culture

The human HCC cell lines Huh7 and Hep3B and normal liver cells (THLE-3) were obtained from the American Type Culture Collection (ATCC). Huh7 and Hep3B cells were cultured in low glucose Dulbecco's Modified Eagle medium (DMEM) (Life Technologies Australia Pty Ltd, #11885084) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 1% antibiotic and antimycotic solution (Life Technologies Australia, #15240062) in a humidified incubator (5% $CO_2$, 37 °C). The immortalised human liver cell line (THLE-3) was grown in bronchial epithelial cell growth (BEBM) medium (Lonza) supplemented with the additive accompanying BEGM Bullet Kit #CC3170 (Lonza), except gentamycin/amphotericin (GA) and epinephrine, and further supplemented with 5 ng/mL human epidermal growth factor (EGF), 70 ng/mL of phosphoethanolamine and 10% FBS. The flask was pre-coated with a mixture of a solution of 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I and 0.01 mg/mL bovine serum albumin dissolved in BEBM basal medium before subculturing of THLE-3 cells.

## 1.3 Backbone synthesis

Poly(HEMA-*ran*-GMA) was synthesised as reported previously.[1] Briefly, monomers were dissolved in methanol (MeOH) (1:3 v/v ratio) and degassed using the freeze/pump/thaw (FPT) technique, then copper(I) bromide (CuBr, 100 mg 0.70 mmol) was combined with 2,2′-bipyridine (bpy, 392 mg, 2.5 mmol) and monomer solutions at various feed ratios (as detailed in Table S1). 2-(4-Morpholino)ethyl 2-bromoisobutyrate (Me-Br) (1 mmol, 210 µL) initiator was added, and the reaction was carried out at 80 °C under standard Schlenk conditions for 2 h before the reaction was opened to air, and an additional MeOH (15mL) added. The product was collected by repeated precipitation in diethyl ether and centrifuged; the

solid product was dried overnight under vacuum. Molecular weight and polydispersity index (PDI) of polymers were measured using gel permeation chromatography (GPC) (Table S2).

**Table S1:** Monomer feed ratios for ATRP reactions to synthesis linear backbone of various monomers ratio.

| | **Feed ratio** | |
|---|---|---|
| | GMA/MeOH | HEMA/MeOH |
| **1a** | 4.61 mL (38.0 mmol) | 0.27 mL (2.0 mmol) |
| **1b** | 4.12 mL (34.0 mmol) | 0.82 mL (6.0 mmol) |
| **1c** | 3.64 mL (30.0 mmol) | 1.36 mL (10.0 mmol) |

## 1.4 Dendron synthesis and attachment

PAMAM dendron synthesis was adapted from published procedures.[2]

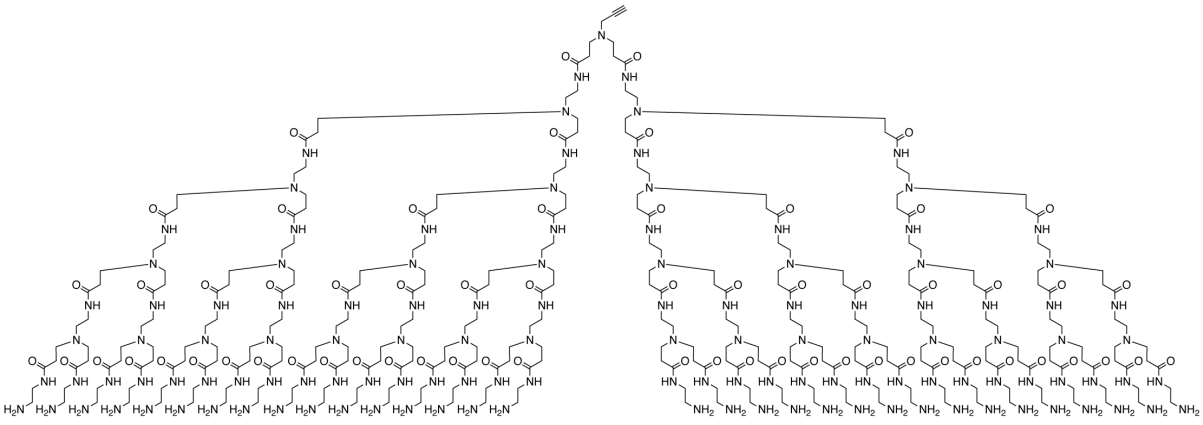

**Figure S1.** Structure of propargyl generation 5.0 PAMAM dendron.

*Azido functionalisation:* Copolymers were functionalised with azido groups as described previously.[1] Briefly, copolymer **1** (1.5 g, 0.34 mmol epoxide) was dissolved in dimethylformamide (DMF, 30 mL). Sodium azide (45 eq) and ammonium chloride (41 eq) were added to the stirred solution and the reaction heated to 60 °C for 72h, then cooled and centrifuged to remove solid. The resulting product was obtained by repeated precipitation in ether from MeOH and dried under vacuum.

*Click reaction:* Generation 4.5 (G4.5) propargyl dendron (320 mg, 110 µmol, 2.4 eq) was dissolved in DMF (15 mL), then added to azido-functionalised copolymer (200 mg, 45.3 µmol azides) dissolved in DMF (5 mL), followed by addition of pentamethyldiethylene triamine (PMDETA, 22.4 µL, 0.11 mmol). The solution was degassed (freeze-pump-thaw 3×), backfilled with argon, then copper(I) bromide (CuBr) (15.2 mg, 0.11 mmol) added and the reaction left stirring under Ar for 72 h at room temperature. Upon completion, deionised (DI) water (10 mL) was added to the reaction vessel and the solution dialysed against DI water (4 × 4 L) for 24h. The product was obtained by lyophilisation, then converted to amine functionalised G5 dendronised

polymer by dissolving in MeOH (5 mL), then dropwise addition to a solution of excess ethylene amine (5 mL) in MeOH (5 mL) at 0 °C. The reaction was left stirring at room temperature for 7 d, then diluted with water and dialysed against DI water (4 × 4 L) before being lyophilised to afford the product as a white solid. Amounts were scaled accordingly for other copolymer and G5 dendrons (method adapted from Zhao *et al*.[3]).

### 1.5 Guanidinobenzoic acid functionalisation of dendronised polymer

4-Guanidinobenzoic acid (GBA) attachment to dendronised polymer was adapted from the method described by Chang *et al*.[4] Briefly, GBA (18.25 mg), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (9.75 mg), and *N*-hydroxysuccinimide (NHS) (16.25 mg) were dissolved in 1 mL of DMF. The solution was stirred at room temperature for 6 h. Triethylamine (a few drops) and dendronised polymer (20 mg) dissolved in PBS were added to the reaction mixture. The reaction pH was adjusted to 8 and left stirring for 5 d at room temperature. Upon completion, the reaction was dialysed against distilled water (4 × 4 L), then lyophilised to afford the product as a white solid.

### 1.6 SP94 functionalisation of dendronised polymer

25% 5G GBA-modified polymer **4** (8.7 mg) was dissolved in 400 µl PBS pH 7.0 and reacted with the heterobifunctional crosslinker SM(PEG)$_{12}$ (2.19 µl, 250 mM in dry DMSO) at room temperature for 2 h. Then, 1 mg SP94 peptide or control peptide (300 µl) was added to the reaction mix and left overnight. The product was purified by dialysis (12-14 kDa MWCO) before being lyophilised.

### 1.7 Instrumental characterisation methods

$^1$H NMR spectra were measured using a Bruker Avance IIIHD 600 MHz spectrometer, with $CD_3OD$ as the solvent for dendronised polymers and $D_2O$ for GBA-modified polymer. The chemical shifts were referenced to the solvent peak, $\delta$ = 3.31 ppm for $CD_3OD$ and $\delta$ = 4.79 ppm for $D_2O$. GPC was used to determine the molecular weight and polydispersity index of polymers (Waters Styragel HR 4 DMF 4.6 × 300 mm column, 5 µm). Agilent Technologies 1100 Series GPC and Agilent GPC software were used for measurements and data analysis, respectively. Measurements were taken using DMF as the eluent at a flow rate of 0.3 mL/min at 50 °C and calibrated against poly(methyl methacrylate) standard.

### 1.8 Polyplex characterisation

Polymers stock solutions were made to a concentration of 5 mg/mL in sterile milli-Q water. For size and zeta potential measurements polymer solutions were mixed with pDNA (1 µg) at an appropriate N/P ratio and incubated at room temperature for 30 min. The intensity-weighted hydrodynamic radius and zeta potential of polyplexes was determined using dynamic light scattering operating at 633 nm; scattering angle of 173°; 4 mW He-Ne laser (Zetasizer Nano ZS, Malvern Instrument UK). Measurements were taken after an initial

equilibration period of 120 s. 'Material' was defined as PGMA (refractive index 1.515 and absorbance 0.05), and 'dispersant' was water at 25 °C (viscosity 0.887 cP and refractive index 1.330).

### 1.9 Transfection

Polymer stock solutions were made to a concentration of 10 mM primary amines in sterile Milli-Q water. Hep3B and Huh7 cells were seeded in standard 12-well plates 12 h before transfection, at concentrations resulting in 30–40% confluency at the time of transfection. Hepatocytes were seeded at 80,000 cells per well in plates treated with the pre-coating mix (described above). Polymer solution and pDNA were diluted to working concentrations in Opti-MEM reduced serum media (Gibco). pDNA solutions were added to the polymer solution and mixed thoroughly to achieve the desired nitrogen to phosphorus (N/P) ratio for 1 µg DNA in a total volume of 130 µL and incubated at room temperature for 30 min. Cells were washed with PBS, and the media was replaced with 300 µL Opti-MEM. Polyplexes (130 µL) were added to the appropriate well containing 300 µL of Opti-MEM and were incubated for 4 h. After a 4 h incubation period, polyplex was removed, and cells were washed with PBS, and the appropriate complete culture medium was added. The experiment was incubated for a further 44 h, giving a total transfection time of 48 h.

Commercial transfection agent FuGENE (Promega Australia) was used according to the manufacturer's protocol at an optimised ratio of 4 µl FuGENE/1 µg pDNA. Transfection efficiency was visualised with representative bright-field and fluorescence microscopy images for each of the conditions tested under epifluorescence microscopy and quantified via flow cytometry (BD LSRFortessa). For flow cytometry quantification, cells were washed twice with 1× phosphate buffered saline (PBS) and harvested with trypsin (Life Technologies). Cells were collected via centrifugation (300*g*, 5 min) and washed in 300 µL FACS buffer (2% FBS, 4 mM EDTA in PBS). Cells were centrifuged again (300*g*, 5 min) and resuspended in 200 µL of FACS buffer for analysis. Data were acquired using BD FACS Diva software. mCherry was excited by 561 nm laser, and emission was measured with 600 nm long pass and 610/20 nm bandpass filters.

### 1.10 Cytotoxicity

Huh7, Hep3B and normal hepatocytes (THLE-3) were seeded in poly(L-lysine)-coated 96-well opaque white plates (Corning) at a density of 5000 cells/well. Cells were allowed to settle for 24 h, the media was removed, cells were washed with PBS and Opti-MEM media containing polyplexes (40 ng pEF1α-mCherry-sgRNA per well) were added. After 4 h, the transfection cocktail was replaced with complete media. Cells were left for another 44 h, and CellTiter-Glo® 2.0 (Promega, 10 µl per well) was added. Plates were shaken and incubated for 10 min following the manufacturer's instructions. The luminescence was recorded (PerkinElmer EnSpire), and the measured value was normalised to untreated controls, pEF1α-mCherry-sgRNA alone and cells treated with Opti-MEM. All conditions were measured in triplicates.

### 1.11 Statistical analysis

Statistical analyses were performed with Graphpad Prism (GraphPad Software Inc.) The data is illustrated as the average; error bars represent the standard deviation. For all tests, differences were considered significant at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Unless otherwise specified, data are represented as the means of technical triplicate and biological duplicate independent experiments.

# 2 Supplemental data

## 2.1 Copolymer characterisation

**Table S2:** Copolymer characterisation as determined by GPC.

| Polymer | $M_w$ / kDa | $Đ$ | GMA mol% by $^1$H NMR integration |
|---|---|---|---|
| **1a** | 15.7 | 1.21 | 5.04% |
| **1b** | 21.5 | 1.30 | 15.7% |
| **1c** | 17.5 | 1.30 | 25.6% |

## 2.2 Dendron attachment

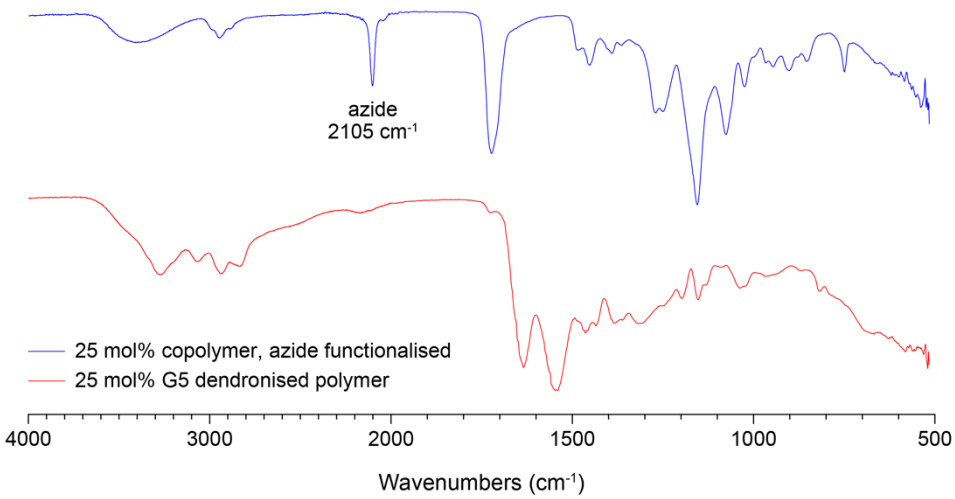


**Figure S2.** FT-IR spectra of 25 mol% azide functionalised copolymer **2c** and G5 dendronised polymer **3c**. The disappearance of the azide peak at 2105 cm$^{-1}$ confirms that the click reaction of dendrons to the copolymer backbone has proceeded to completion. This data is the same as in ref (5).

## 2.3 GBA-modified dendronised polymer polymer characterisation

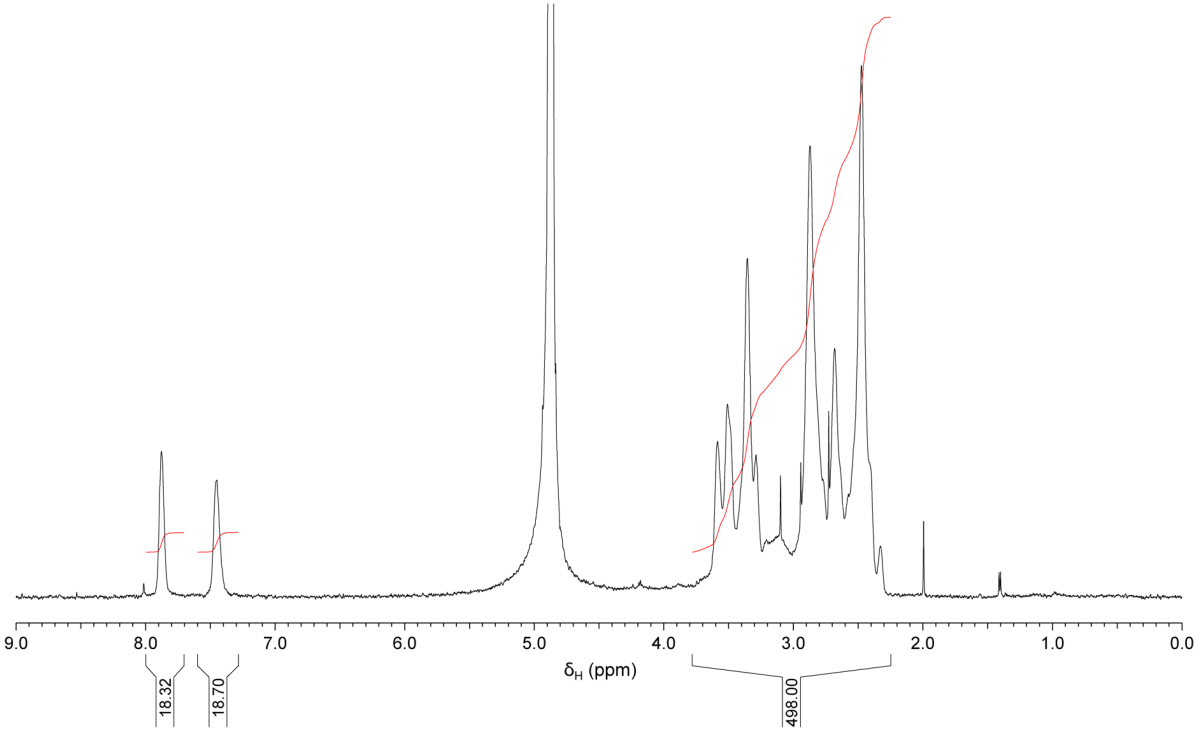


**Figure S3.** $^{1}$H NMR spectra of 25% dendronised polymer bearing 5G PAMAM dendrons terminated with phenylguanidine moiety. Spectrum recorded in $D_2O$ at 600 MHz. The integration gives (18.3+18.7)/4 = 9.3 GBA per dendron, equivalent to 29% modification of dendron peripheral primary amines.

## 2.4 Polyplex characterisation by DLS

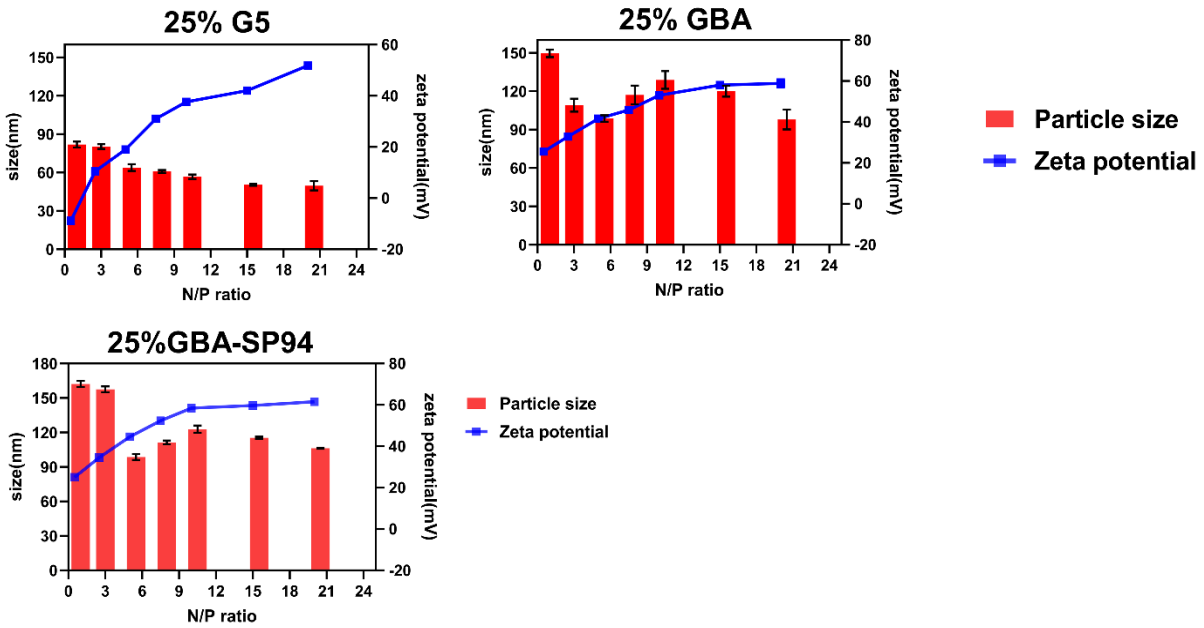


**Figure S4.** DLS and zeta potential optimisation for 25%G5, 25% GBA, 25% GBA-SP94 in milli-Q $H_2O$ adjusted to pH 7 without additional buffers. mCherry plasmid was mixed at different N/P ratio with the polymers.

## 2.5 Optimisation of transfection using GBA-modified dendronised polymer

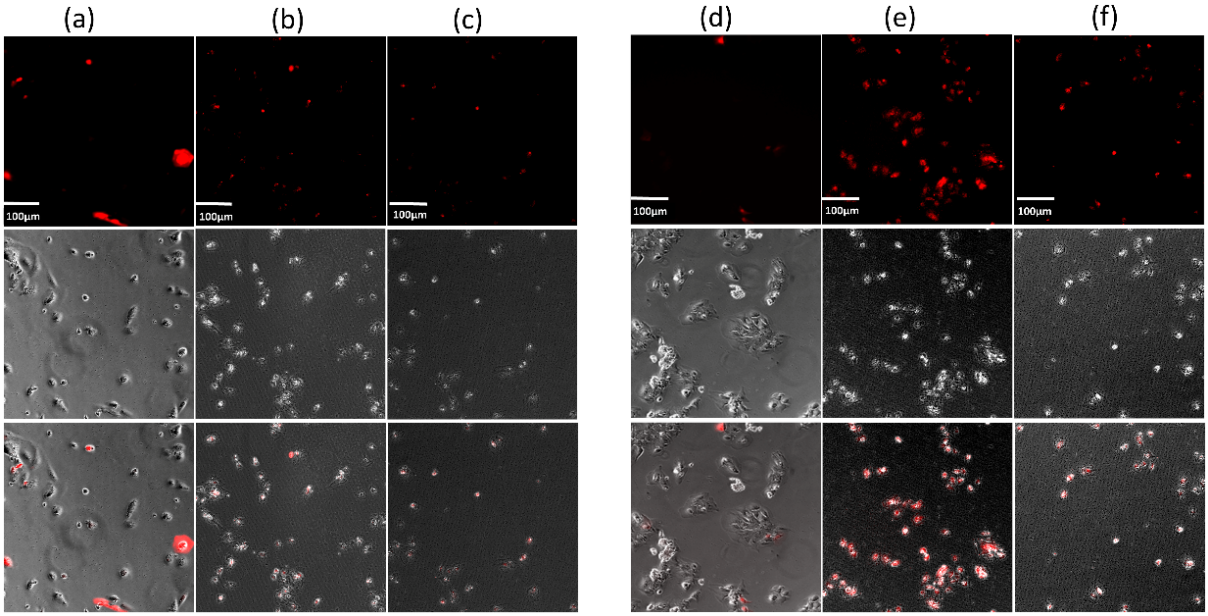


**Figure S5:** Microscopic representative images of PAMAM transfection optimised at N/P (a) 5, b) 10, c) 15 in Hep3B, d) N/P ratio 5, e) 10 and f) 15 in Huh7.

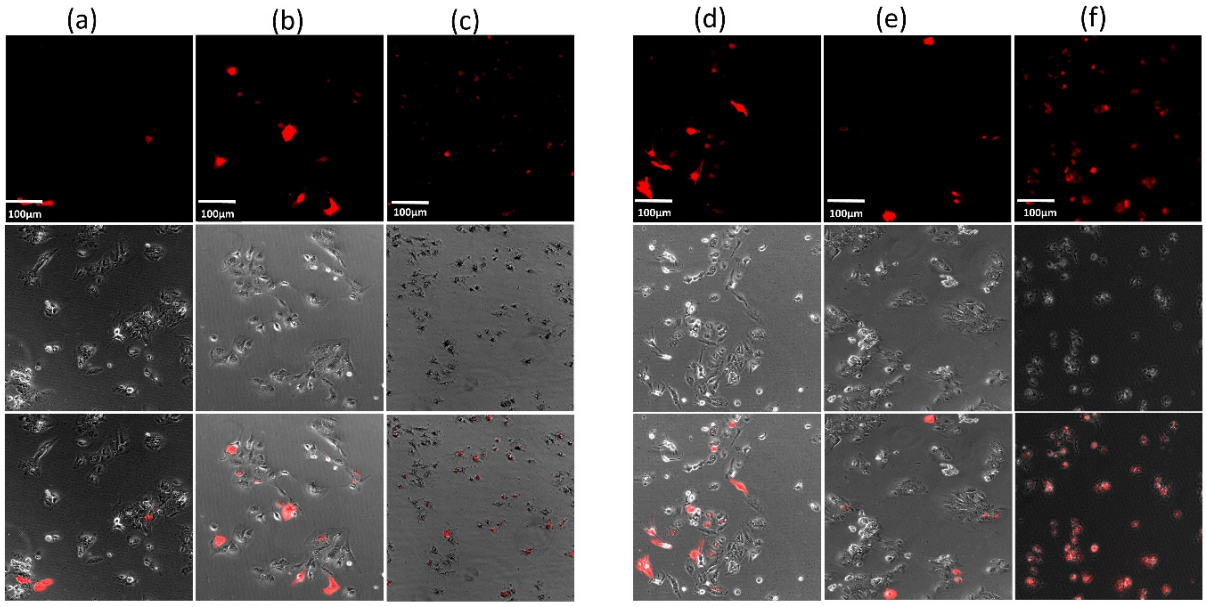


**Figure S6.** Microscopic representative images of 25% G5 transfection optimised at N/P (a) 10, b) 15, c) 20 in Hep3B, N/P ratio d) 10, e) 15 and f) 20 in Huh7.

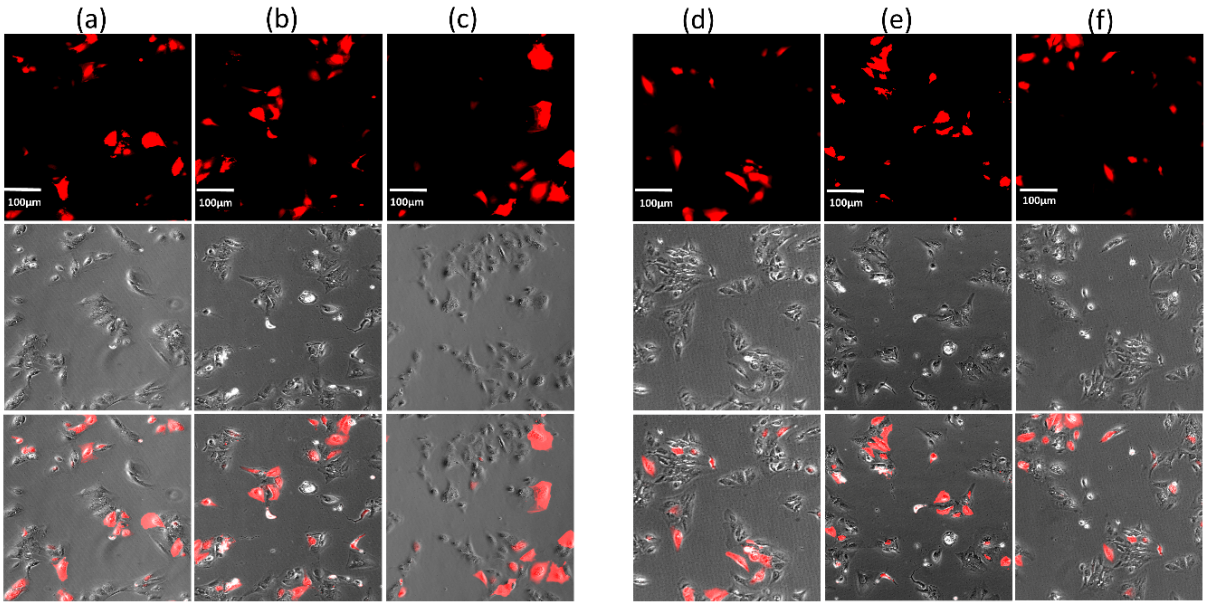


**Figure S7:** Microscopic representative images of 25% GBA transfection optimised at N/P (a) 10, b) 15, c) 20 in Hep3B, N/P ratio d) 10, e) 15 and f) 20 in Huh7.

## 2.6 Cellular uptake of transfection agent

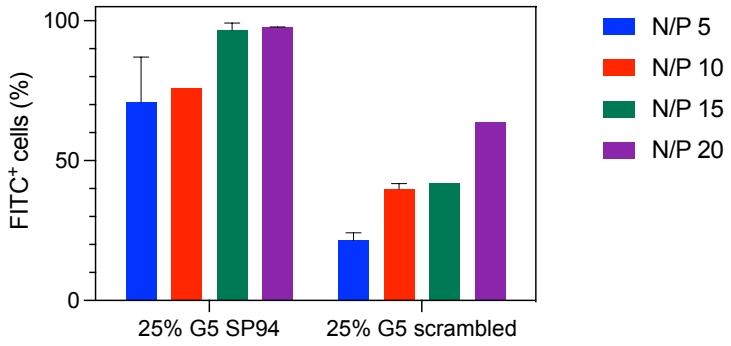


**Figure S8.** Comparison of cellular uptake of 25% G5 polymer modified with FITC-labelled peptide (SP94 or scrambled SP94) in Huh7 cells, as quantified by flow cytometry.

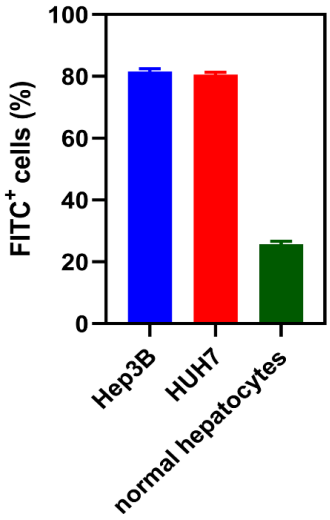


**Figure S9.** Cellular uptake of 25% GBA-SP94 in Hep3b, Huh7 and normal hepatocytes measured by flow cytometry.

## 2.7 Comparison of transfection in normal hepatocytes

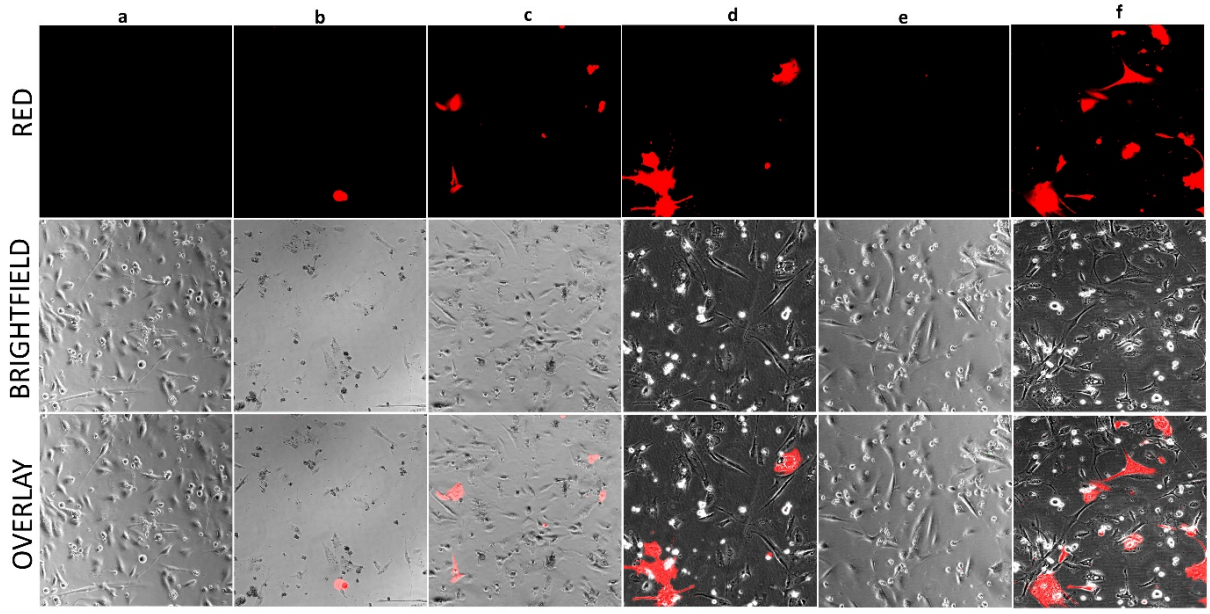


**Figure S10.** Microscopic representative images of mCherry transfection in normal hepatocytes using transfecting agent a) untreated cells b) PAMAM c) 25% G5, d) 25% GBA, e) 25% GBA-SP94 and f) FuGENE.

## 2.8 Cytotoxicity

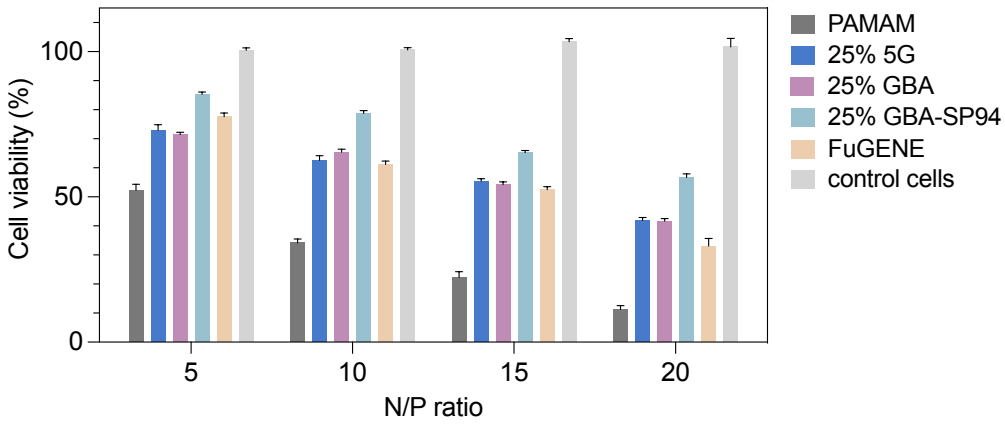


**Figure S11.** Cell viability of Hep3B cells after using different transfecting agents at different N/P ratios.

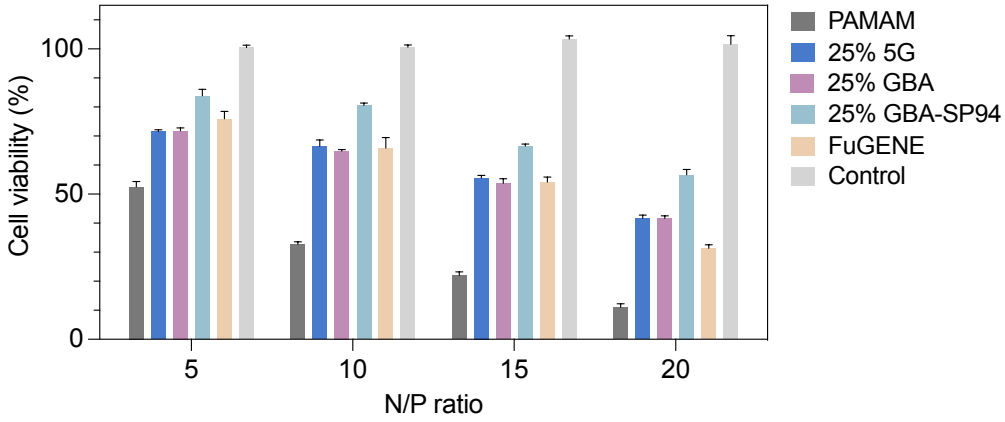


**Figure S12.** Cell viability of Huh7 cells after using different transfecting agents at different N/P ratios.

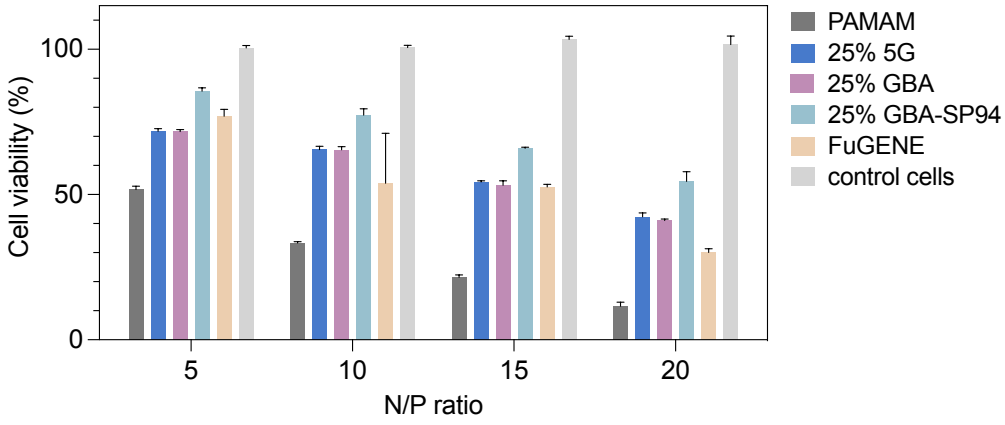


**Figure S13.** Cell viability of normal hepatocytes (THLE-3) after using different transfecting agents at different N/P ratios.

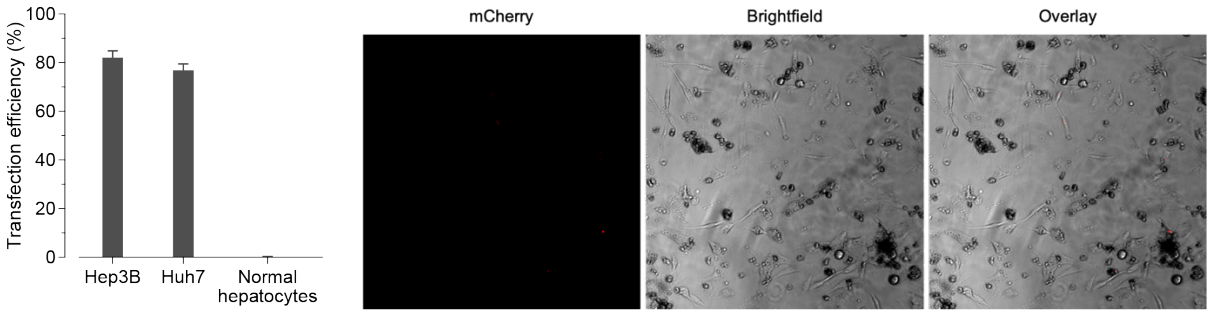


**Figure S14.** High transfection efficiencies were achieved using Lipofectamine 3000 in Hep3B and Huh7 cells, and low efficiencies in THLE-3 normal hepatocytes. However, a representative image of normal hepatocytes following transfection with Lipofectamine 3000 shows the low transfection is due to substantial toxicity rather than selectivity.

## 2.9 Standard curve for SP94 determination

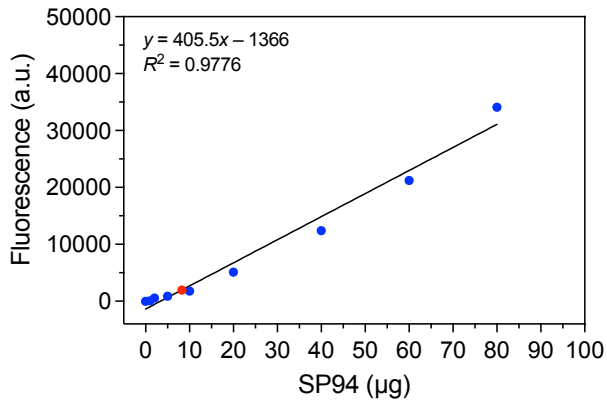


**Figure S15.** Standard curve of FITC-tagged SP94. The fluorescence signal from polymer-conjugated peptide sample (100 µg) is indicated by the red point and was determined as 8.3 µg SP94.

# 3 References


1. Kretzmann JA, Ho D, Evans CW, Plani-Lam JHC, Garcia-Bloj B, Mohamed AE, et al. Synthetically controlling dendrimer flexibility improves delivery of large plasmid DNA. *Chem Sci.* 2017;8(4):2923-30.
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