

Messenger RNA delivery by hydrazone-activated polymers

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Abbreviations

DLS: dynamic light scattering; DMEM: Dulbecco's Modified Eagle Medium; DMF: dimethylformamide; DMSO: Dimethylsulfoxide; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt); EGFP: enhanced green fluorescent protein; FBS: Fetal Bovine Serum; FSC: forward scatter channel; GALA: WEAALAEALAEALAEHLAEALAEALEALAA;¹ hpt: hours post transfection; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PEI: polyethyleneimine; PBS: phosphate-buffered saline; R8: octaarginine;² SSC: side scatter channel; TAE: tris-acetate-ethylenediaminetetraacetic acid.

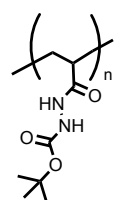
Materials and Methods

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Avance III 300 MHz spectrometer. Chemical shifts are reported in ppm (δ units) referenced to the following solvent signals: DMSO- d_6 δ H 2.50 and D₂O δ H 4.79. Dialysis was carried out in deionised water at room temperature for a minimum of 48 hours using a Spectra/Por 6 1000 Molecular weight cut-off (MWCO) 38 mm width membrane. Gel Permeation Chromatography (GPC) was performed with a Shimadzu Prominence LC-20A fitted with a Thermo Fisher Refractomax 521 Detector and a SPD20A UV-vis Detector. Boc-Protected poly (acryloyl hydrazide) (Boc-P) was analysed using 0.05 M LiBr in DMF at 60 °C, or 0.005 M NH₄BF₄ in DMF at 50 °C, as the eluent and a flow rate of 1 mL min⁻¹. The instrument was fitted with a Polymer Labs PolarGel guard column (50 × 7.5 mm, 5 μ m) followed by two PLGel PL1110–6540 columns (300 × 7.5 mm, 5 μ m). Molecular weights were calculated based on a standard calibration method using polymethylmethacrylate. Poly(acryloyl hydrazide) P was analysed using Dulbecco's Phosphate Buffered Saline 0.0095 M (PO₄) without Ca and Mg as the eluent and a flow rate of 1 mL min⁻¹. The instrument was fitted with an Agilent PL aquagel-OH column (300 × 7.5 mm, 8 μ m) and run at 35 °C. A Guava EasyCyteTM cytometer (EMD Millipore) was used for all flow cytometry experiments. Cell microscopy images were acquired with an Andor Zyla 4.2 digital camera mounted on a Nikon Eclipse Ti-E epifluorescence microscope or with a Leica SP5 confocal microscope. Absorbance of formazan was measured in a microplate reader Tecan Infinite F200Pro. Gels were imaged on a ChemiDoc XRS+ system (Bio-Rad) and analysed with the software ImageLab 5.2.1 (Bio-Rad).

DLS and ζ potential measurements were performed in a Malvern Zetasizer NanoZSP using standard disposable cuvettes. All experiments were done in triplicate at 25 °C.

Polymer synthesis

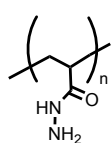
Poly(*tert*-butyl-2-acryloylhydrazine-1-carboxylate) (**Boc-P**)



A solution of 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) (23 mg, 70.0 μ mol) in DMSO (1.5 mL) and a solution of 2-aminoethanethiol (27.6 mg, 360 μ mol) in DMSO (1.5 mL) were added sequentially to a solution of *tert*-butyl-2-acryloylhydrazine-1-carboxylate

(3.3 g, 17.9 mmol) in DMSO (17 mL). A 50 μ L aliquot of this solution was taken at this stage to aid in the calculation of conversion. The reaction mixture was then sealed and degassed with Argon for 30 min. The degassed solution was left to react at 65°C for 5 h. The reaction was stopped by allowing it to cool down to room temperature and by exposing it to air. A 50 μ L aliquot of this solution was taken at this stage to aid in the calculation of conversion. The polymer was purified by dialysis against water. The water was removed by lyophilisation and by drying in a desiccator with P₂O₅ to afford 2.86 g Boc-P as an off-white powder (quantitative yield). UV (DMSO): λ_{max} 300 nm. ¹H-NMR (300 MHz, DMSO-*d*₆) δ (ppm): 9.9-7.4 (br-m, 2H, NH), 2.4-0.5 (br, 12H, 9H in C(CH₃)₃, 3H in CHCH₂). Conversion: 86%; Mn (DMF GPC) 196 KDa; Đ (DMF GPC) 2.76.

Poly(acryloyl hydrazide) P



Trifluoroacetic acid (TFA) (4 mL) was added dropwise to poly(*tert*-butyl-2-acryloylhydrazine-1-carboxylate) (Boc-P) (499 mg) and the yellow solution was stirred at r.t. overnight. Excess of TFA was removed by blowing a steady stream of Argon and the resulting oil was diluted in water (4 mL). The P·TFA salt formed was neutralised by adding NaHCO₃ until no foaming was observed.

The colourless solution was allowed to stir overnight. The crude polymer was purified by dialysis against water. The water was removed by lyophilisation and by drying in a desiccator with P₂O₅ to afford 60 mg of P as a white powder (26%). ¹H-NMR (300 MHz, D₂O) δ (ppm): 1.2–2.3 (br m, (3·DP)H). Mw (Aqueous GPC) > 59 KDa.

Supporting figures

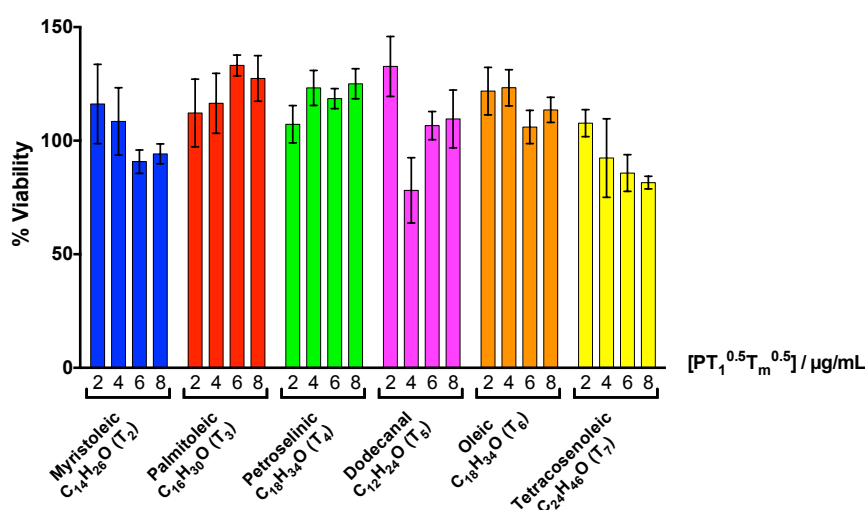


Figure S1. MTT viability assay. Hek293 cells were transfected with the indicated concentrations of the polyhydrazones and 1 ng/ μ L of mRNA, and 24 hpt cell viability was measured with a MTT colorimetric assay. Values were normalized to untreated cells.

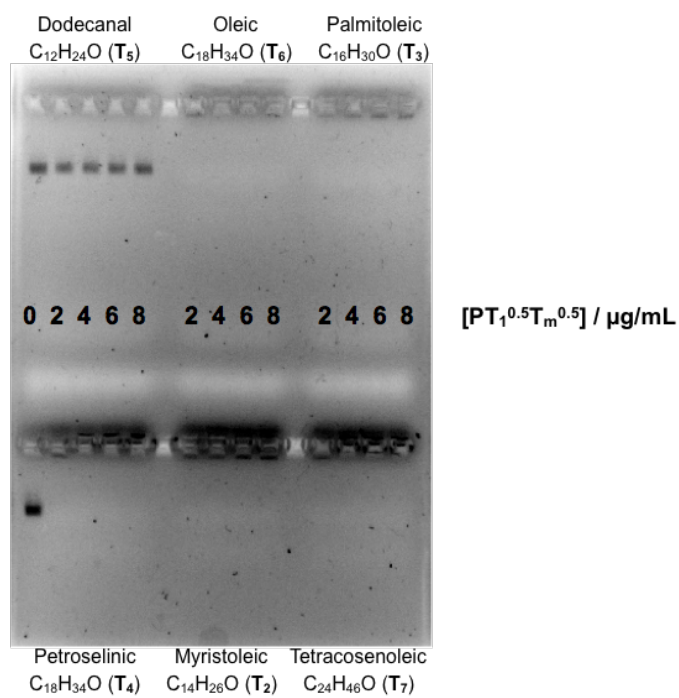


Figure S2. Gel retardation assay with the six different polyhydrazones. **EGFP-mRNA** and $PT_1T_m/EGFPmRNA$ polyplexes with different concentrations of polyhydrazones: 2, 4, 6 and 8 $\mu\text{g/mL}$.

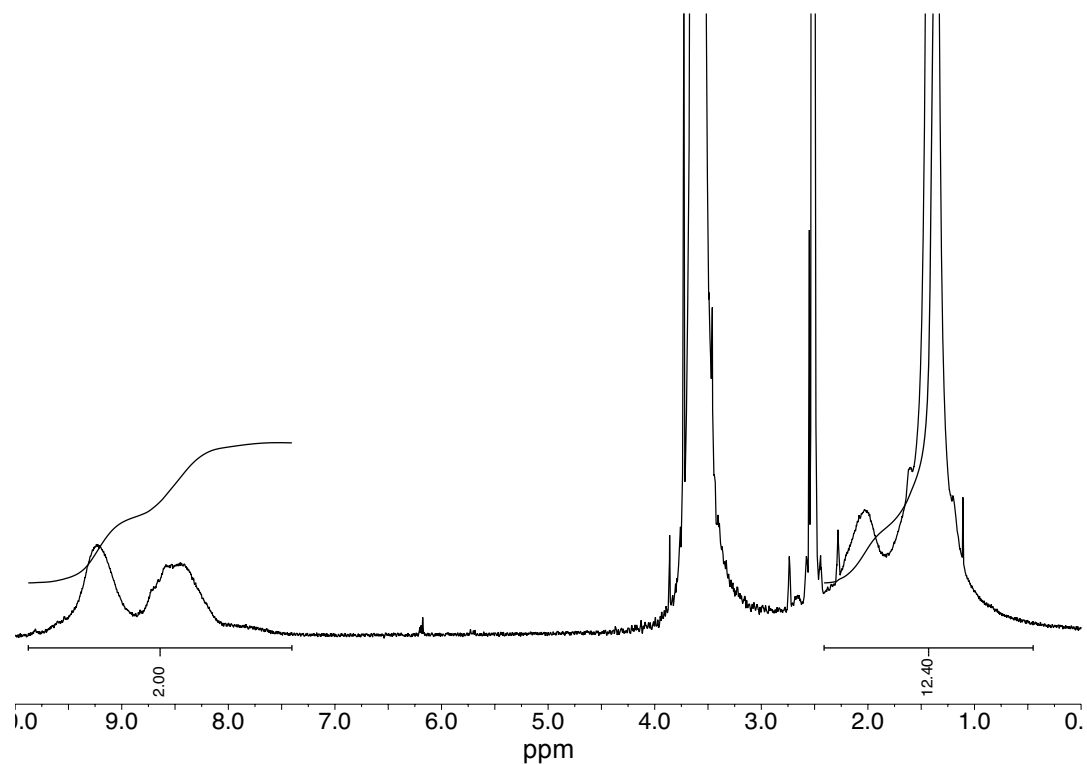


Figure S3. $^1\text{H-NMR}$ spectra for Boc-P in $\text{DMSO-}d_6$.

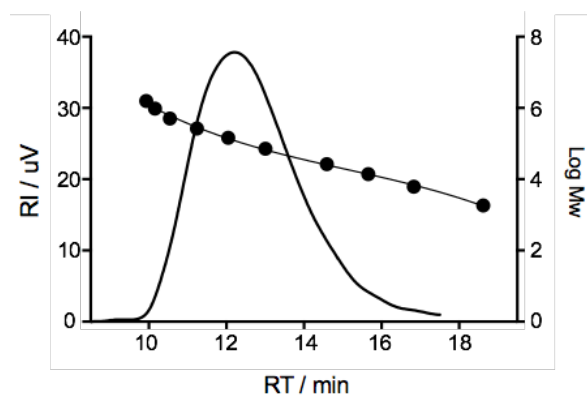


Figure S4. GPC Chromatogram of Boc-P in DMF.

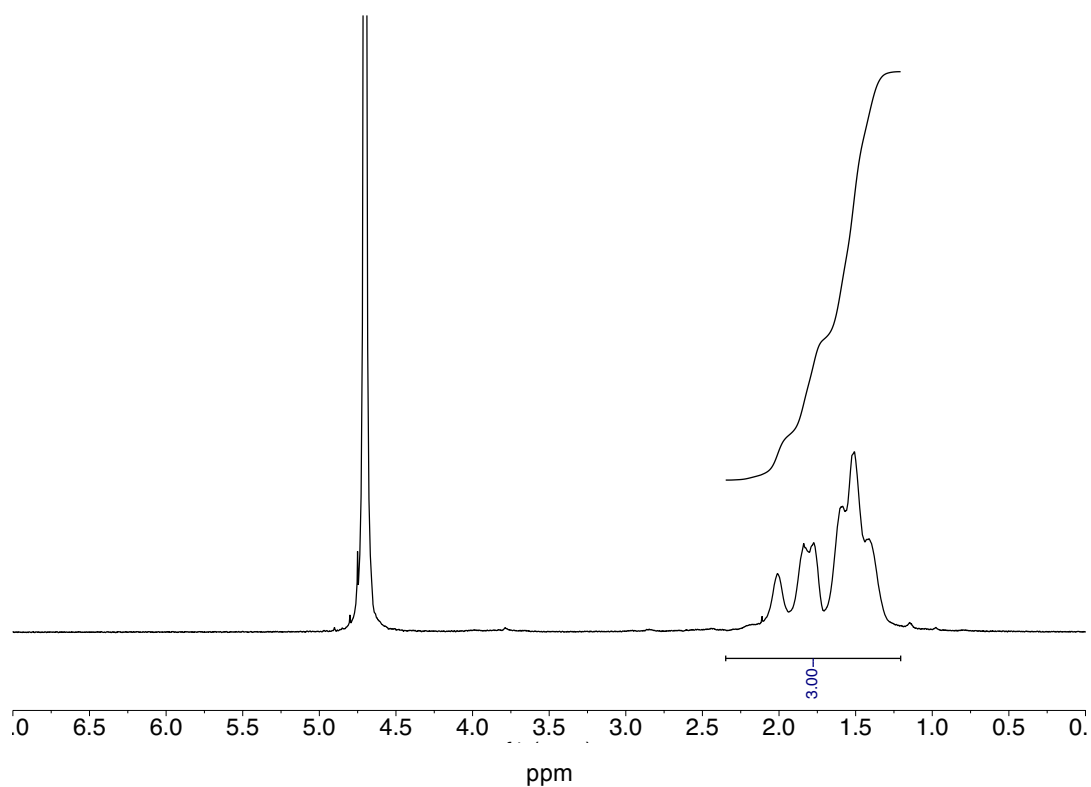


Figure S5. ^1H -NMR spectra for P in D_2O .

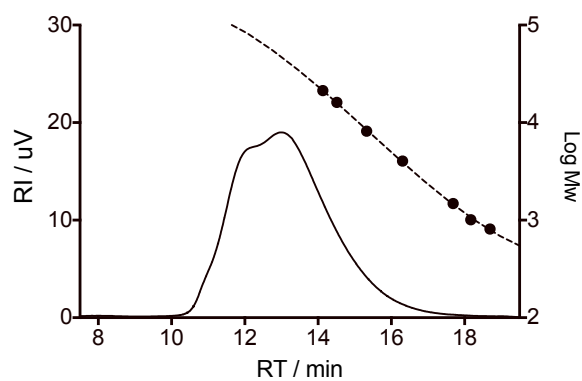


Figure S6. GPC Chromatogram of P in DPBS.

Supporting references

- 1 R. A. Parente, S. Nir and F. C. Szoka, *Biochemistry*, 1990, **29**, 8720–8728.
- 2 M. Juanes, I. Lostalé-Seijo, J. R. Granja and J. Montenegro, *Chem. Eur. J.*, 2018, **24**, 10689–10698.