Influence of block versus random monomer distribution on the cellular uptake of hydrophilic copolymers

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SUPPORTING INFORMATION
1. Experimental Procedures

1.1. Materials
Acetone, dimethyl sulfoxide (DMSO), diethyl ether, 1,8-Diazabicycloundec-7-ene (DBU), sodium borohydride, thiazolyl blue tetrazolium bromide (MTT), 1,4-dioxane, 4,4′-azobis(4-cyanopentanoic acid) (ACVA), N,N-dimethylacrylamide (DMA), 4-acyrloylmorpholine (NAM), N-(2-Hydroxyethyl)acrylamide (HEAm) and Sephadex G-15 were obtained from Sigma Aldrich. (Propanoic acid)yl butyl trithiocarbonate (PABTC) was received from DuluxGroup Australia and used as received. Fluorescein-5-maleimide was obtained from TCI chemicals. N,N′-dimethylformamide (DMF) was obtained from VWR chemicals. 1,4-Dioxane was distilled under reduced pressure prior to use. Milli-Q water was used as solvent for polymerizations. DMA was filtered through a basic aluminium oxide (activated, basic, Brockmann I, standard grade, ∼150 mesh, 58 Å) column to remove the radical inhibitor. All polymerization were carried out under argon atmosphere.

Dulbecco’s Modified Eagle’s Medium (DMEM), Phosphate Buffered Saline (PBS), Roswell Park Memorial Institute medium (RPMI), Penicillin-Streptomycin, Trypsin and Fetal Bovine Serum (FBS) were obtained from Life Technologies. All reagents were use as received unless otherwise specified.

1.2. Methods & Instruments
Molar mass distributions were measured using size exclusion chromatography (SEC) on an Agilent GPC50 liquid chromatography system, using DMF + 0.1% LiBr as eluent and a Polar Gel-M guard column (50 × 7.5 mm) and two Polar Gel-M columns (300 × 7.5 mm). Poly(methyl methacrylate) (PMMA) standards were used to calibrate the SEC system. Samples were filtered through a polytetrafluoroethylene (PTFE) membrane with 0.2 µm pore before injection. Experimental molar mass ($M_n$,SEC) and $M_w/M_n$ (Ð) values of synthesized polymers were determined by conventional calibration using Cirrus software. Details about the calculations of monomer conversions, $M_n$,th and the theoretical number fraction of living chains can be found here.¹

Flow cytometry data were obtained on a BD Accuri C6 Flow Cytometer at excitation 488 nm and emission 530 nm with band-pass of 30 nm. Raw data was analysed using Tree Star FlowJo X 10.0.7r2 software.

DLS data was obtained on a Malvern Zetasizer Nano ZS in 40 uL cuvettes at 25 °C. Samples were measured at a concentration of 120 µM. Each sample was analyzed three times and the average of the results is reported. Fluorescence and absorbance values were measured using a plate reader Infinite 200 Pro (Tecan). UV-Vis measurements were carried out on a Cary 100 Bio spectrometer from Varian using 10 mm quartz cuvettes. A cuvette filled with PBS was used to obtain a baseline and each measurement was obtained using a double-beam.

1.3. Co-polymers synthesis
Polymers were prepared via RAFT polymerization using methods previously described.²,³ In a typical synthesis, the initial block of copolymer was prepared by introducing PABTC, monomer, ACVA and H₂O:dioxane (80:20) as the solvent in a sealed flask with a magnetic stirrer. The flask was then degassed by bubbling argon through the solution for 15 min, before initiating the RAFT polymerization by heating the mixture at 70 °C. After 24 h, addition of the next block was carried out by adding a further mixture of degassed monomer, ACVA and solvent to the polymerization medium, and allowing the mixture to polymerize at 70 °C for a further 24 h. Statistical polymer R was prepared
using similar polymerization conditions but by mixing all three monomers at the onset of the polymerization instead.

1.4. Polymer Functionalization
Each copolymer was functionalized with a fluorescein dye in a one-pot reaction. In a typical reaction, copolymer (50 mg, 6.8 µmol), fluorescein-5-maleimide (4.5 mg, 10.5 µmol) and NaBH₄ (0.0053 g, 140.1 µmol) were dissolved in 1 mL DMF and purged with argon for 5 minutes in the absence of light. The mixture was then left to stir at room temperature overnight, following which a catalytic quantity of DBU (10 µg, 0.1 µmol) previously dissolved in 100 µL of DMF was added. The reaction was then left to stir for 6 days in the absence of light. The mixture was then precipitated in cold acetone and filtered on a fritted glass filter. The precipitate was dissolved in MilliQ water and freeze-dried. Further purification of functionalized polymers was done using a combination of dialysis and water-based Sephadex G-15 column.

1.5. Cell Lines
HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 100 µg of penicillin-streptomycin/mL. A2780 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 100 µg of penicillin-streptomycin/mL.

1.6. Cytotoxicity Assay
Cell viability was tested using standard protocol for the MTT assay. Briefly, HeLa cells were seeded in a 96 well plate at a density of 2.5 × 10⁴ cells per well. After 16 hours, the culture medium was replaced by fresh media containing a series of dilution of the polymers (250, 50, 10, 2 and 0.4 µg/mL), prepared from polymer stock solutions in PBS at 1 mg/mL and previously filtered through a 0.22 µm syringe filter. Following 24 hours incubation, the medium was removed and the cells washed with PBS. The cells were incubated with a solution of MTT in phenol red- and FBS-free medium (0.45 mg/mL) for 3.5 hours. The supernatant was then removed and formazan crystals left to dissolve in DMSO for 15 minutes at 37 °C. Absorbance of the samples was finally measured using a plate reader at 570 nm.

1.7. Cellular uptake experiments
Uptake of the fluorescein-copolymer conjugates was measured using standard flow cytometry protocols. HeLa or A2780 cells were seeded in a 24 well plate at a density of 3.5 × 10⁵ cells per well. After 16 hours, the culture medium was replaced by fresh media containing the polymers (either 50 µg/mL or corrected concentrations), prepared from polymer stock solutions in PBS at 1 mg/mL previously filtered through a 0.22 µm syringe filter. Cells were then incubated for either 2 hours or 24 hours, following which the medium was removed, cells washed with PBS twice, trypsinised, centrifuged, re-dispersed in cold PBS with 3% FBS and filtered into a FACS tube for analysis. Cells were kept on ice prior to measurement to limit exocytosis of the compounds. The geometric mean of the fluorescence was used as the sample value. The data presented in Figure 2 are representative of two independent experiments where each sample was measured in triplicate. Errors reported always correspond to the standard deviation of the mean. Statistical analysis was performed using Student’s t test with (*) p < 0.1, (**) p < 0.01, (***) p < 0.001.
2. Additional Figures

Table S1. Relative fluorescence of fluorescein-modified copolymers in PBS and fluorescence correction of flow cytometry geometric mean.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fluorescence [a.u.]$^a$</th>
<th>Correction Factor $^b$</th>
<th>A2780, 2 hours</th>
<th>HeLa, 2 hours</th>
<th>HeLa, 24 hours</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>original</td>
<td>corrected</td>
<td>original</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
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</table>

$^a$ Fluorescence values at 50 µg/mL, as measured using graph from Figure 2

$^b$ Fluorescence[polymer]/Fluorescence[fluorescein]

Figure S1. Dynamic light scattering measurement of copolymer chain sizes in PBS.
Figure S2. Comparison between UV spectra of the copolymer prior fluorescein attachment (peak at 310 nm corresponds to CTA group) and the fluorescence spectra of fluorescein-functionalized copolymers (peak at 490 nm corresponds to fluorescein emission).

Figure S3. Comparison between the uptake profiles of various co-polymers using a) 50 μg.mL⁻¹ of polymers, fluorescence average corrected using fluorescence factor; b) 48.85 μg.mL⁻¹ of A, 62.50 μg.mL⁻¹ of B, 60.65 μg.mL⁻¹ of C and 43.63 μg.mL⁻¹ of R (equal fluorescence intensity for each solutions).

3. References

(2) Gody, G.; Barbey, R.; Danial, M.; Perrier, S. Polymer Chemistry 2015, 6, 1502.