Cell-penetrating poly(disulfide)s: Dependence of activity, depolymerization kinetics and intracellular localization on their length

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Supplementary Information

Table of Content

1. Materials and Methods

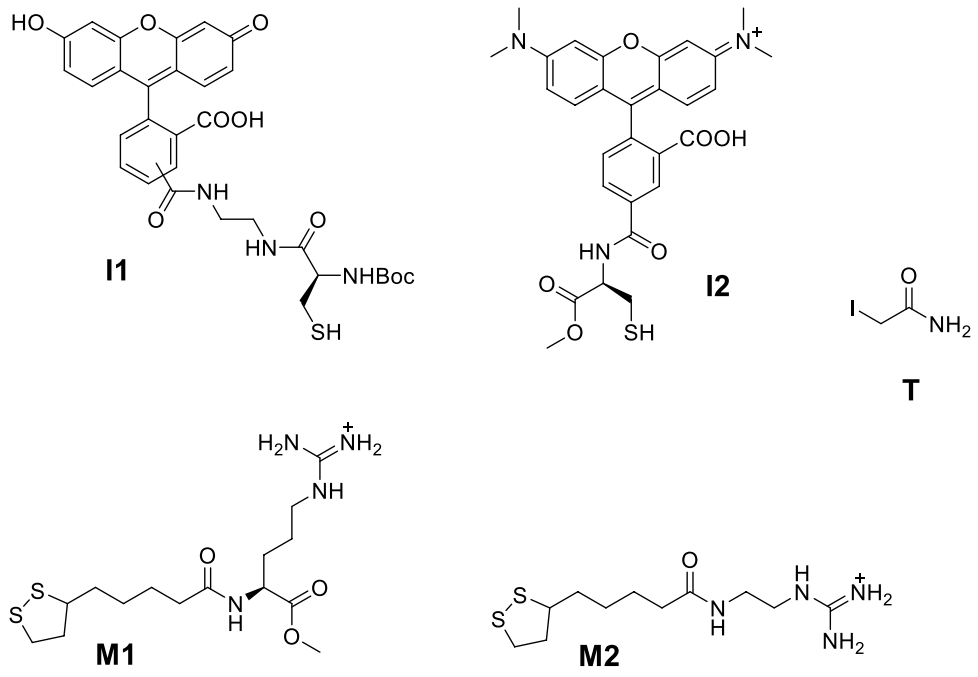
As in ref. S1, Supporting Information. Briefly, reagents for synthesis were purchased from Fluka, Sigma-Aldrich, TCI and Across, buffers and salts of the best grade available from Fluka or Sigma-Aldrich and used as received. Egg yolk phosphatidylcholine (EYPC) were purchased from Avanti Polar Lipids.

Fluorescence measurements were performed with a FluoroMax-4 spectrofluorometer (Horiba Scientific) equipped with a stirrer and a temperature controller (25.0 ± 0.1 ºC). Fluorescence spectra were corrected using instrument-supplied correction factors. UV-Vis spectra were recorded on a JASCO V-650 spectrophotometer equipped with a stirrer and a temperature controller (25.0 ± 0.1 ºC) and are reported as maximal absorption wavelength λ in nm (extinction coefficient ε in $M^{-1}cm^{-1}$). Gel-permeation chromatography (GPC) was performed using a JASCO LC-2000Plus system equipped with quaternary pump (JASCO PU-2089), photodiode array (JASCO MD-2018 Plus) and fluorescence (JASCO FP-2020 Plus) detectors. The chromatographic column used was a Superdex 75 10/300 GL (flow 0.4 mL/min, eluent: 30 % ACN in 0.1 M acetate buffer pH = 6.5). pH values were measured with a Consort C832 multi-parameter analyzer equipped with a VWR glass membrane pH electrode calibrated with Titrisol solution from Merck at pH 4.00 and 7.00. Vesicles were prepared with a Mini-Extruder from Avanti Polar Lipids (pore size 100 nm).

Abbreviations. ACN: Acetonitrile; CF: 5(6)-Carboxyfluorescein; DMF: *N*,*N*-Dimethylformamide; EDTA: Ethylenediaminetetraacetic acid EYPC: Egg yolk phosphatidylcholine; GPC: Gel permeation chromatography; GSH: Glutathione; LUVs: Large unilamellar vesicles; MeOH: Methanol; PDI: Polydispersity index; rt: Room temperature; TAMRA: 5-Carboxytetramethylrhodamine TEOA: Triethanolamine; Tris: Tris(hydroxymethyl)aminomethane.

2. Synthesis

All synthesis procedures and product characterizations are reported in ref S2 and S3.



Scheme S1. Polymer building blocks.

3. Polymerization

3.1. General Procedure

The procedure described in ref. S2 was adapted and slightly modified to obtain each polymer. Stock solutions of propagators (400 mM in DMF), initiators (**I1** or **I2**, 100 mM in DMF) and terminator (iodoacetamide, 500 mM in water) were freshly prepared and fluxed with N_2 gas. Appropriate amounts of **M1** or **M2** were transferred to an Eppendorf tube and dried *in vacuo*. TEOA buffer (1 M, pH = 7.0) and the initiator solution were added, yielding the desired final concentration of the reaction mixture. The sample was kept at 25 °C with vigorous agitation. After the required polymerization time, the reaction mixture was quenched upon 20 times dilution with the terminator solution, yielding 10 mM overall guanidinium cations. Specific conditions for each polymer are reported in Table S1. The

obtained mixture was purified by GPC within a day. The obtained stock solutions of the polymers were kept at -20 °C.

Table S1. Polymerization conditions

Poly(disulfide)s	Monomer (mM)	Initiator (mM)	TEOA *c* (M) / pH	Time (min)
$\mathbf{1}^{\mathbf{20.2}}$	200	4	0.65 / 7.0	60
$\mathbf{1}^{\mathbf{8.3}}$	175	5	0.65 / 7.0	30
$\mathbf{1}^{\mathbf{8.1}}$	200	5	0.65 / 7.0	30
$\mathbf{1}^{\mathbf{7.7}}$	160	5	0.65 / 7.0	30
$\mathbf{1}^{\mathbf{6.9}}$	200	5	0.65 / 7.0	20
$\mathbf{1}^{\mathbf{5.7}}$	150	5	0.65 / 7.0	30
$\mathbf{1}^{\mathbf{4.1}}$	150	5	0.65 / 7.0	30
$\mathbf{1}^{\mathbf{1.9}}$	150	5	0.65 / 7.0	10
$\mathbf{2}^{\mathbf{5.0}}$	200	4	0.65 / 7.0	60
$\mathbf{2}^{\mathbf{4.3}}$	200	5	0.65 / 7.0	30
$\mathbf{2}^{\mathbf{3.2}}$ [a]	200	5	0.65 / 7.0	30
$\mathbf{2}^{\mathbf{3.1}}$ [a]	200	5	0.65 / 7.0	30
$\mathbf{2}^{\mathbf{1.1}}$	200	5	0.65 / 7.0	10

[a]Obtained from the same polymerization mixture, two fractions were collected during GPC purification: $\mathbf{2}^{\mathbf{3.2}}$ R_t = 29-34.5 min, $\mathbf{2}^{\mathbf{3.1}}$ R_t = 34.5-41 min

3.2. Purification and Characterization

Poly(disulfide)s were purified by GPC, using Superdex 75 10/300GL (10×300 mm) chromatographic column and 30% ACN in 0.1 M acetate buffer (pH = 6.5) as eluent. After termination, the polymerization mixture was diluted with the eluent. The final concentrations of guanidinium cations in the samples were 5 mM ~ 7.5 mM. Sample (100 μL) was loaded on the column and the flow rate was set to 0.4 mL/min from 0 to 40 min, 0.6 mL/min from 41 to 80 min. Absorbance at 220 and 333 nm were used for detection. Fluorescence detection was also used, λ_{ex} = 492 nm and λ_{em} = 517 nm for initiator **I1**, λ_{ex} = 552 nm and λ_{em} = 582 nm for initiator **I2**. The polymer peak was collected, lyophilized and the resulting solid was dissolved in water and desalted through Sephadex G-25 gel-filtration resin. Quantification of the resulting polymer solution was achieved by UV-Vis measurements, using initiator **I1** or **I2** to generate calibration curves. The polymer concentration of the obtained stock solutions

were ranging between 10 and 150 μM, as confirmed by vesicle assay. Molecular weight of each polymer was determined by analytical injection in GPC, using molecular weight standards for calibration. Results are summarized in Table S2 and S3.

Table S2. Characterization and transport activities in LUVs of purified poly(disulfide)s based on monomer **M1**

Entry	M_n[a]	M_w[b]	PDI	EC_{50}[c]	Y_{max}[d]	*Hill Coeff.n*
$\mathbf{1}^{\mathbf{20.2}}$	19.2	20.2	1.06	20.8 ± 6.8	84.2 ± 5.0	1.2 ± 0.5
$\mathbf{1}^{\mathbf{8.3}}$	6.9	8.3	1.21	9.5 ± 1.1	86.7 ± 3.0	2.6 ± 0.7
$\mathbf{1}^{\mathbf{8.1}}$	6.3	8.1	1.30	48.1 ± 7.6	67.1 ± 6.4	1.5 ± 0.3
$\mathbf{1}^{\mathbf{7.7}}$	6.5	7.7	1.19	20.7 ± 1.5	92.3 ± 2.6	3.4 ± 0.7
$\mathbf{1}^{\mathbf{6.9}}$	4.8	6.9	1.43	37.6 ± 2.8	62.2 ± 2.7	1.8 ± 0.2
$\mathbf{1}^{\mathbf{5.7}}$	4.7	5.7	1.20	73.2 ± 11.6	73.6 ± 5.4	1.7 ± 0.4
$\mathbf{1}^{\mathbf{4.1}}$	3.2	4.1	1.29	102.6 ± 11.7	58.5 ± 4.0	1.4 ± 0.2
$\mathbf{1}^{\mathbf{1.9}}$	1.7	1.9	1.16	253.0 ± 60.8	67.0 ± 5.0	1.2 ± 0.2

[a]Number average molecular weight (kDa) from GPC profiles. [b]Mass average molecular weight (kDa) from GPC profiles. [c]EC_{50} expressed as polymer concentration (nM), calculated from Hill analysis of dose response curves using equation Eq S2. [d]Maximum transport activity (%).

Table S3. Characterization and transport activities in LUVs of purified poly(disulfide)s based on monomer **M2**

Entry	M_n[a]	M_w[b]	PDI	EC_{50}[c]	Y_{max}[d]	*Hill Coeff.n*
$\mathbf{2}^{\mathbf{5.0}}$	3.5	5.0	1.44	16.0 ± 1.3	97.5 ± 2.1	2.4 ± 0.4
$\mathbf{2}^{\mathbf{4.3}}$	2.9	4.3	1.48	13.3 ± 1.2	96.4 ± 2.2	2.1 ± 0.4
$\mathbf{2}^{\mathbf{3.2}}$	1.5	3.2	2.16	12.6 ± 1.1	92.9 ± 2.5	1.8 ± 0.2
$\mathbf{2}^{\mathbf{3.1}}$	2.1	3.1	1.45	13.9 ± 1.1	90.3 ± 2.5	2.1 ± 0.3
$\mathbf{2}^{\mathbf{1.1}}$	0.9	1.1	1.14	23.3 ± 1.3	96.5 ± 1.6	2.2 ± 0.2

[a]Number average molecular weight (kDa) from GPC profiles. [b]Mass average molecular weight (kDa) from GPC profiles. [c]EC_{50} expressed as polymer concentration (nM), calculated from Hill analysis of dose response curves using equation Eq S2. [d]Maximum transport activity (%).

4. Properties of Cell-Penetrating Poly(disulfide)s

4.1. Vesicle Preparation

LUVs were prepared following the general procedures in refs. S4-S6. A thin lipid film was obtained by evaporating a solution of 25 mg EYPC in 1 mL MeOH/$CHCl_3$ 1:1 on a rotary evaporator (rt) and then *in vacuo* overnight. The resulting film was hydrated with 1.0 mL buffer (50 mM CF, 10 mM Tris, 10 mM NaCl, pH 7.4) for more than 30 min, subjected to freeze-thaw cycles (5×) and extrusions (15×) through a polycarbonate membrane (pore size, 100 nm). Extravesicular components were removed by gel filtration (Sephadex G-50) with 10 mM Tris, 107 mM NaCl, pH 7.4 buffer as eluent. Final conditions: ~5 mM EYPC; inside: 50 mM CF, 10 mM Tris, 10 mM NaCl, pH 7.4; outside: 10 mM Tris, 107 mM NaCl, pH 7.4.

4.2. Transport Activity in Fluorogenic Vesicles

EYPC-LUVs stock solutions (25 μL) were diluted with buffer solution (10 mM Tris, 107 mM NaCl, pH 7.4), placed in a thermostated fluorescence cuvette (25 °C) and gently stirred (total volume in the cuvette, 2000 μL; final lipid concentration, ~62.5 μM). CF efflux was monitored at λ_{em} 517 nm (λ_{ex} 492 nm) as a function of time after addition of polymers solutions (25 μL) at t = 50 s and 1.2% aqueous triton X-100 (40 μL, 0.024% final concentration) at t = 300 s. Fluorescence intensities were normalized to fractional emission intensity I(t) using equation (Eq S1):

$$I(t) = (I_t - I_0) / (I_\infty - I_0) \qquad \text{(Eq S1)}$$

where $I_0 = I_t$ just before reaction mixtures addition, $I_\infty = I_t$ at saturation after lysis. Effective concentration for polymers or monomers EC_{50} and Hill coefficient n were determined by plotting the fractional activity Y (= I(t) at saturation just before lysis, t = ~290 s) as a function of polymer concentration c and fitting them to the Hill equation (Eq S2)

$$Y = Y_0 + (Y_{MAX} - Y_0) / \{1 + (EC_{50} / c)^n\} \qquad \text{(Eq S2)}$$

where Y_0 is Y without polymer or monomer, Y_{MAX} is Y with an excess polymer at saturation, EC_{50} is the concentration of polymer required to reach 50% activity and n is the Hill coefficient. Results are summarized in Table S2 and S3.

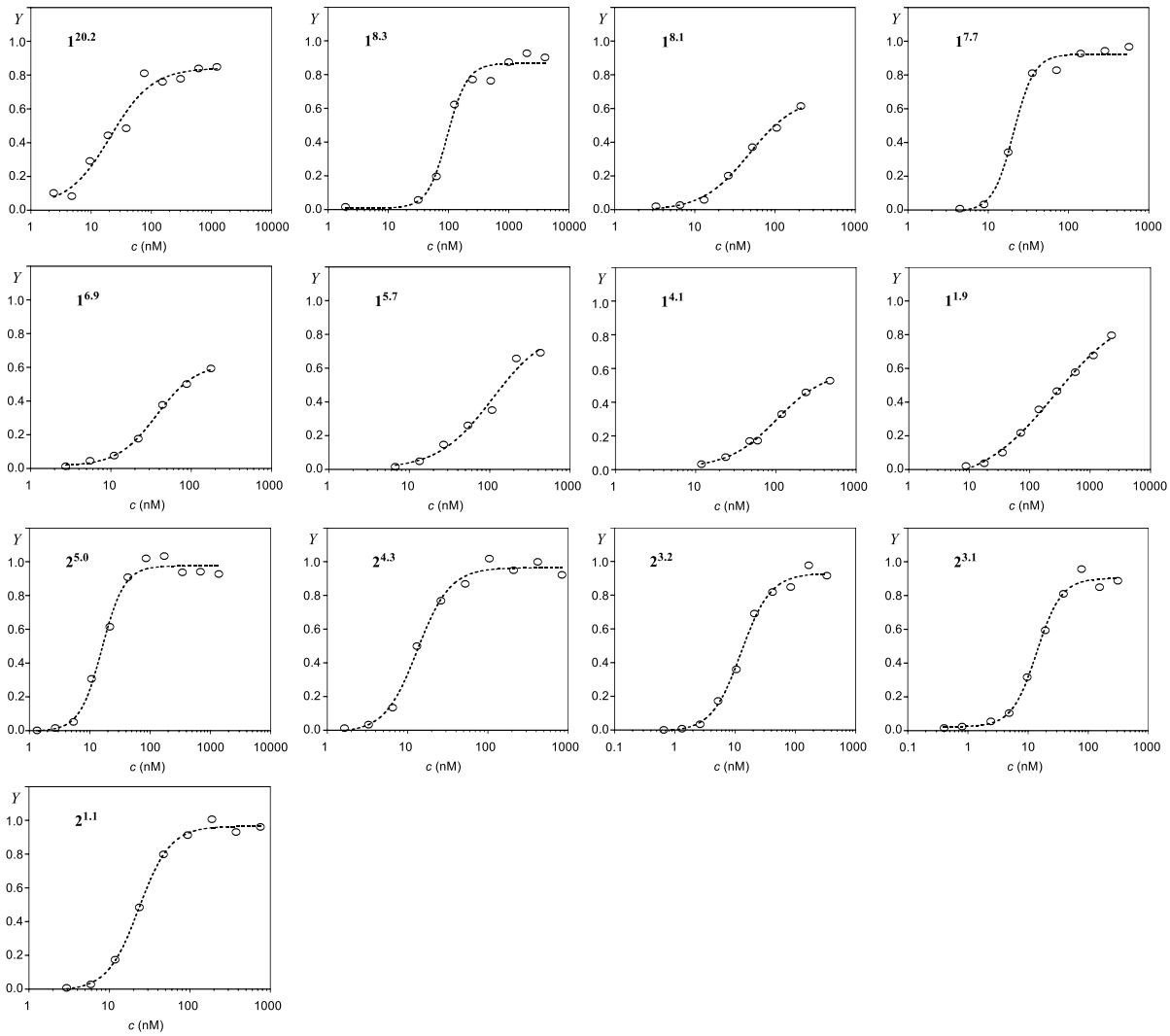


Figure S1. Transport activity *Y* in EYPC-LUVs⊃CF for the polymers used in this study.

5. Depolymerization Kinetics

The depolymerization assay was performed for polymers with concentration higher than 20 μM and with an EC_{50} lower than 100 nM

5.1 Depolymerization Assay

To study the depolymerization by reduction with GSH, a 500 nM solution of polymer was prepared in 0.1, 0.2, 0.4, 0.5, 0.75, 1, 1.5, 2.5 or 5 mM solution of reduced GSH (10 mM Tris,

107 mM NaCl, 1mM EDTA, pH 7.4). After 1, 7, 13, 19, and 30 min of incubation at 37 °C an aliquot of the reaction mixture (100 μL for polymer $\mathbf{1}^{20.2}$, $\mathbf{1}^{8.3}$, $\mathbf{1}^{7.7}$, $\mathbf{2}^{5.0}$, $\mathbf{2}^{4.3}$, $\mathbf{2}^{3.2}$, $\mathbf{2}^{3.1}$, 300 μl for polymer $\mathbf{1}^{5.7}$) was added to the EYPC⊃CF vesicle solution (25 μL) diluted with buffer (10 mM Tris, 107 mM NaCl, pH 7.4), placed in a thermostated fluorescence cuvette (25 °C) and gently stirred (total volume in the cuvette, 2000 μL; final lipid concentration, ~62.5 μM). CF efflux was monitored at λ_{em} 517 nm (λ_{ex} 492 nm) as a function of time after addition of reaction mixtures at t = 50 s and 1.2% aqueous triton X-100 (40 μL, 0.024% final concentration) at t = 300 s. Fluorescence intensities were normalized to fractional emission intensity I(t) using Equation (Eq S1):

$$I(t) = (I_t - I_0) / (I_\infty - I_0) \qquad \text{(Eq S1)}$$

where $I_0 = I$ of a blank of EYPC-LUVs (25 μL) diluted with buffer (10 mM Tris, 107 mM NaCl, pH 7.4) and reaction mixture without polymer (100 μL for polymer $\mathbf{1}^{20.2}$, $\mathbf{1}^{8.3}$, $\mathbf{1}^{7.7}$, $\mathbf{2}^{5.0}$, $\mathbf{2}^{4.3}$, $\mathbf{2}^{3.2}$, $\mathbf{2}^{3.1}$, 300 μL for polymer $\mathbf{1}^{5.7}$, X mM GSH, 10 mM Tris, 107 mM NaCl, 1 mM EDTA pH 7.4) for a total volume of 2000 μL in the cuvette. $I_\infty = I_t$ at saturation after lysis. The depolymerization half-time was determined by plotting the fractional activity Y (= I(t) at saturation just before lysis, t = ~290 s) as a function of incubation time and fitting with the Hill equation (Eq S3)

$$Y = Y_0 + (Y_{MAX} - Y_0) / \{1 + (t_{50} / t)^n\} \qquad \text{(Eq S3)}$$

where Y_0 is Y without polymer or monomer, Y_{MAX} is Y with an excess polymer or monomer at saturation, t is the incubation time, t_{50} is the time required to reach 50% activity of polymer and n is the Hill coefficient. The t_{50} values for the polymers tested are reported in Table S4.

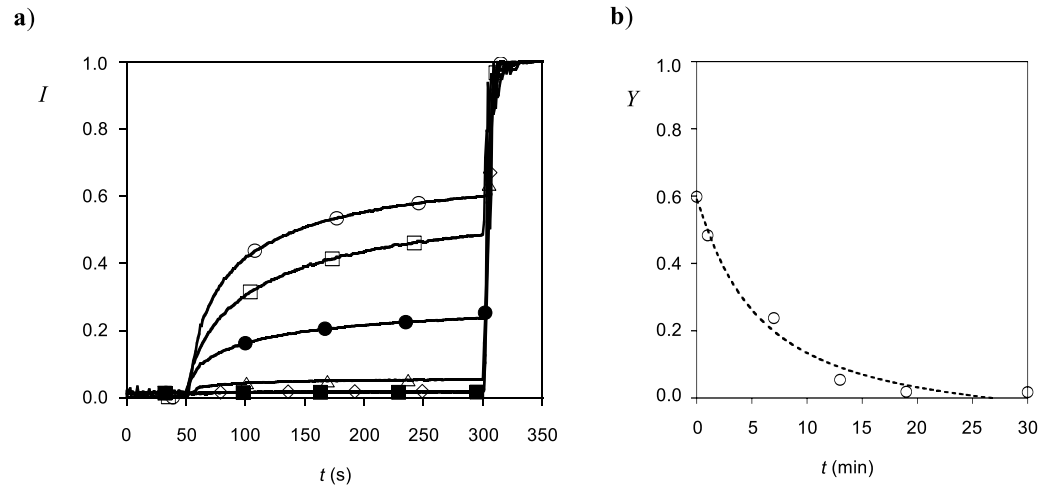


Figure S2. (a). Representative change in CF emission intensity *I* during the addition to EYPC-LUVs⊃CF of $\mathbf{1}^{\mathbf{20.2}}$ before (○) and after (□) 1, (●) 7, (Δ) 13, (◊) 19, (■) 30 minutes incubation with 0.75 mM GSH. (b) Corresponding maximal transport activity *Y* plot in function of the incubation time with 0.75 mM GSH, the dotted line corresponds to the fitting with Hill equation to determine the t_{50}.

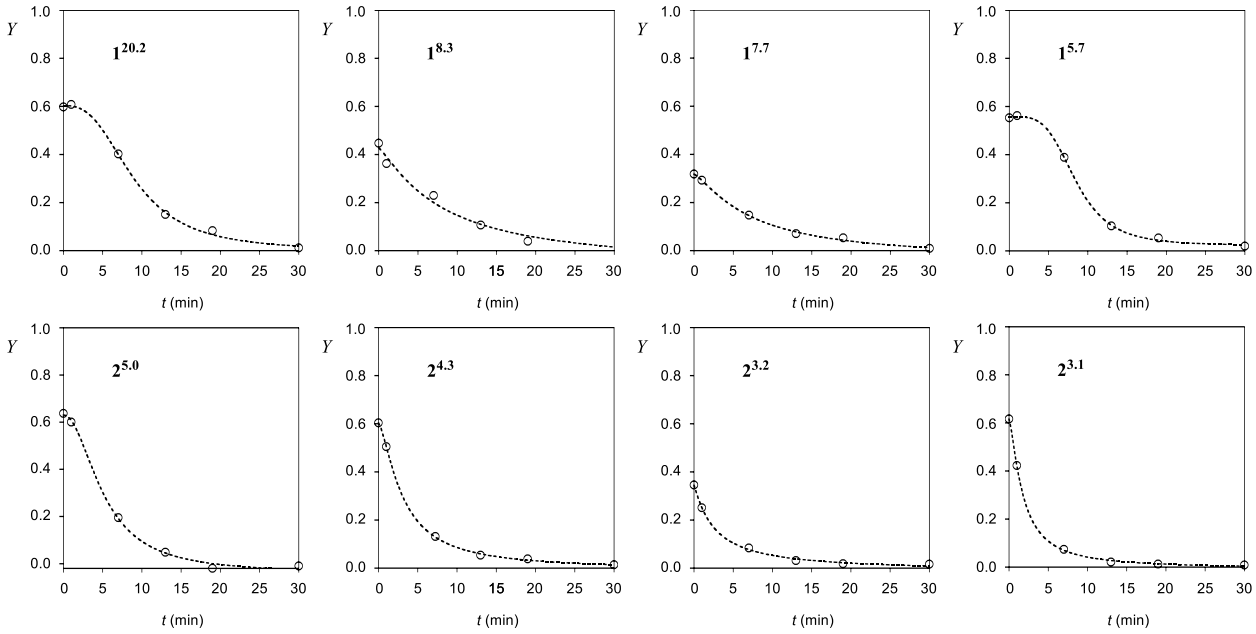


Figure S3. Representative transport activity *Y* in EYPC-LUVs⊃CF for the polymers used in this study after different incubation time with [GSH] = 0.375 mM.

Table S4. t_{50} (s) in function of the concentration of GSH

Poly(disulfide)s	Concentration of GSH (mM)								
	5	2.5	1.5	1	0.75	0.5	0.375	0.1875	0.1
$\mathbf{1}^{\mathbf{20.2}}$	66.03	85.40	113.00	217.07	331.69	390.07	539.15	966.30	2746.74
$\mathbf{1}^{\mathbf{8.3}}$	32.16	61.86	88.16	114.74	203.04	426.02	483.99	818.16	2061.30
$\mathbf{1}^{\mathbf{7.7}}$	53.82	65.79	94.31	94.66	211.34	126.99	442.23	1569.12	674.22
$\mathbf{1}^{\mathbf{5.7}}$	8.99	67.90	292.11	326.43	347.39	299.14	510.23	633.30	686.76
$\mathbf{2}^{\mathbf{5.0}}$	N.A.[a]	78.47	78.70	134.11	183.37	336.62	308.77	562.16	1146.36
$\mathbf{2}^{\mathbf{4.3}}$	N.A.[a]	56.00	60.55	106.15	94.97	203.16	212.27	442.81	511.37
$\mathbf{2}^{\mathbf{3.2}}$	N.A.[a]	34.90	66.59	72.60	138.38	172.36	156.98	273.97	503.52
$\mathbf{2}^{\mathbf{3.1}}$	N.A.[a]	39.14	76.21	86.38	138.08	102.29	263.75	312.67	916.98

[a]Not attributed

5.2 Determination of the Depolymerization Rate Constant

The depolymerization rate constant and the order of reaction was determined by plotting the logarithm of the half-time as a function of the logarithm of the concentration of GSH and fitting with the linear regression equation (Eq S4)

$$log(t_{50})=(1-n)log([GSH])+log\{(2^{n-1}-1)/(k_d(n-1))\} \quad \text{(Eq S4)}$$

where n is the order of reaction and k_d is the depolymerization rate constant. Results are summarized in Table S5.

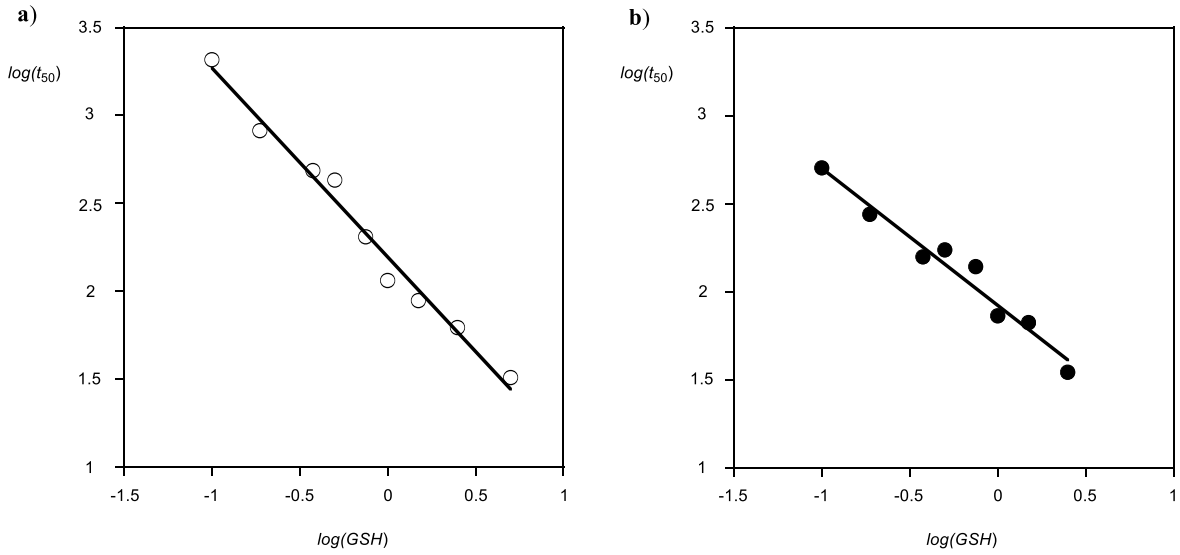


Figure S4. Graphical example of the logarithm of t_{50} in function of the logarithm of the concentration of GSH and their linear regression curves (Eq. S4) for (a) polymer $\mathbf{1}^{\mathbf{8.3}}$ and (b) polymer $\mathbf{2}^{\mathbf{5.0.}}$

Table S5. Depolymerisation rate constant and order of reaction for polydisulfides **1** and **2**		
Entry	*Order of reaction n*	*Depolymerization rate constant k* ($s^{-1}M^{-1}$).
$\mathbf{1}^{\mathbf{20.2}}$	1.96	4.462
$\mathbf{1}^{\mathbf{8.3}}$	2.07	6.393
$\mathbf{1}^{\mathbf{7.7}}$	1.84	6.807
$\mathbf{1}^{\mathbf{5.7}}$	2.02	7.187
$\mathbf{2}^{\mathbf{5.0}}$	1.87	6.961
$\mathbf{2}^{\mathbf{4.3}}$	1.77	10.086
$\mathbf{2}^{\mathbf{3.2}}$	1.78	11.954
$\mathbf{2}^{\mathbf{3.1}}$	1.89	11.070

6. Cellular Uptake Experiments

6.1. Cell Culture

Human cervical cancer-derived HeLa-Kyoto cells were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 1% Penicillin / Streptomycin

(PS) and 1% L-Glutamine. The cells were grown on a 25 cm^3 tissue culture flask (TPD corporation) at 37 °C under 5% CO_2.

6.2. Confocal Microscopy

HeLa-Kyoto cells were seeded at 6 × 10^4 cells/well on 35 mm glass-bottomed dishes (MatTek Corporation) and cultured overnight. After removing the medium, the cells were washed twice with PBS and treated with 1 mL of polymer solution (500 nM in Leibovitz's medium). The cells were incubated for 15 min at 37 °C, then the media was removed by aspiration. Cells were washed 3 times with PBS containing 0.1 mg/mL heparin and the cells were kept in Leibovitz's medium. Distribution of fluorescent polymers was analyzed without fixing using a confocal laser scanning microscope (Leica SP5) equipped with 63× oil immersion objective lens. Ar laser was used as light source (6% laser power) with excitation wavelength 488 nm and emission 498 - 535 nm for CF–labelled polymers (Leica HyD™ detector); DPSS laser (8% laser power) with excitation 561 nm and emission 571 - 610 nm for TAMRA-labelled polymers (Leica HyD™ detector). During CLSM analysis the sample was kept at 37 °C.

7. Supplementary References

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