ADVANCED HEALTHCARE MATERIALS

Supporting Information

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PH-Triggered, Lymph Node Focused Immunodrug Release by Polymeric 2-Propionic-3-Methyl-maleic Anhydrides with Cholesteryl End Groups

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PH-TRIGGERED, LYMPH NODE FOCUSED IMMUNODRUG RELEASE BY POLYMERIC 2-PROPIONIC-3-METHYLMALEIC ANHYDRIDES WITH CHOLESTERYL END GROUPS

-Supporting Information-

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1. Chemicals and Solvents

All reagents and solvents were purchased from TCI Chemicals (Tokyo, Japan), Sigma Aldrich (Taufkirchen, Germany) or Rapp Polymers (Tübingen, Germany) and used as received, unless otherwise described. Oxalyl chloride was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Benzylamine, dibenzylamine, triethylamine, dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), chloroform ($CHCl_3$), dichloromethane (DCM) and all deuterated solvents were bought from Sigma Aldrich. N-(3-aminopropyl)methacrylamide hydrochloride (APMA) was purchased from abcr GmbH (Karlsruhe, Germany) and tetramethylrhodamine cadaverine from Biotium (Fermont, CA, USA).

Immunodrug 1-(4-(aminomethyl)benzyl)-2-butyl-1*H*-imidazo[4,5-c]quinoline-4-amine (IMDQ) was synthesized by Maximilian Scherger (Max Planck Institute for Polymer Research, Germany).[1–3] Azobisisobutyronitrile (AIBN) was recrystallized from ethanol twice.

Hexafluoroisopropanol (HFIP) was obtained from Fluorochem Ltd. (Hadfield, UK). Millipore (mp) water was prepared using a MILLI-Q® Reference A+ System. For silica gel chromatography silica with particle size of 0.063-0.2 mm from Macherey-Nagel GmbH & Co. KG (Dueren, Germany) was used.

Dulbecco's phosphate-buffered saline (PBS), cell culture medium and supplements were bought from Thermo Fisher Scientific. The RAW-Dual™ (IRF-Lucia/KI-[MIP-2]SEAP) murine macrophage reporter cell line and the QUANTI-Blue™ solution were obtained from InvivoGen (San Diego, CA, USA). RAW-Dual™ cells were cultured in Dulbecco's modified Eagle's medium DMEM, containing 10% fetal bovine serum, 1% penicillin/streptomycine, 0.02% normocine, and 0.01% zeocin at 37 °C with 5% CO_2 saturation.

2. Instrumentation

2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H, ^{13}C, ^{19}F and 2D NMR spectra were recorded on a Bruker Avance III 300 MHz, Bruker Avance III 400 MHz, Bruker Avance III 500 MHz or Bruker Avance III 700 MHz spectrometer at room temperature. Diffusion ordered spectroscopy (DOSY) were measured on a Bruker Avance III 500 MHz or Bruker Avance III 700 MHz spectrometer. Calibration of the spectra was achieved using the solvent signal. NMR spectra were analyzed using MestReNova 14.2.0 by Mestrelab Research.

2.2 Size Exclusion Chromatography (SEC)

Analytical SEC was performed in HFIP as an eluant, containing 3 g/L potassium trifluoroacetate at a flow rate of 0.8 mL/min at 40 °C. Measurements were carried out on a SECcurity2instrument from PPS, Mainz equipped with SECcurtity2isocratic pump, a degasser, an autosampler, a column thermostat, an UV and RI detector. The column material was composed with modified silica gel (PFG columns, particle size 7 µM, porosity: 100 Å and 1000 Å), obtained from PSS Polymer Standards Service GmbH, Mainz, Germany. The molecular weights were determined by using a calibration with poly(methyl methacrylate) (PMMA) standard, purchased from PSS, Mainz. Polymer samples were prepared at 1 mg/mL and filtered through GHP syringe filters (0.2 µm pore size, Acrodisc) prior to injection.

2.3 Dynamic Light Scattering (DLS)

Single-angle dynamic light scattering measurements were performed on a Malvern Z Nano instrument equipped with a He-Ne-Laser (λ = 632.8 nm) using the ZetaSizer Software 7.12. All samples were measured in triplicates at 25 °C and a detection angle of 173°. The resulting data were processes by cumulant fitting for D_z and PDI, or by CONTIN fitting for intensity-, volume- and number-weighted particle size distribution. Samples were prepared at 0.1 mg/mL and dust was removed by filtration through GHP syringe filters (0.2 µm pore size, Acrodisc).

2.4 Ultraviolet-Visible Spectroscopy (UV-Vis) and Fluorescence Spectroscopy

UV-Vis spectra were recorded using a Thermo Scientific™ NanoDrop™ 2000c spectrophotometer with a Hellma Quartz Cuvette.

Fluorescence spectroscopy as well as RAW-blue and MTT assay absorbance read-out were performed using a Spark 20M Multimode Microplate Reader from Tecan Trading AG (Mannedorf, Switzerland).

2.5 Mass Spectrometry (ESI-MS and MALDI-ToF)

ESI mass spectra were measured on an Agilent 6545 QTOF-MS (Santa Clara, CA, USA). Samples were dissolved in methanol with a concentration of 0.1 mg/mL. Mass spectrometry data were analyzed using Advion Data Express software.

Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-ToF) measurement were acquired on a rapifleX^{TM} MALDI-ToF/ToF mass spectrometer from Bruker Daltonik GmbH (Bremen, Germany). The instrument was fitted with a 10 kHz scanning smartbeam Nd:YAG laser at 355 nm wavelength and a 10 bit 5 GHz digitizer. Mass spectra were recorded in a positive ion mode using DCTB (trans-2-[3-(4-tbutylphenyl)-2-methyl-2-propenylidene]-malononitrile) acid as a matrix. Samples were prepared at 0.1 mg/mL and the resulting data were processed by mMass software.

2.6 Fluorescent Confocal Microscopy

Fluorescent confocal laser scanning microscopy images were recorded on a Leica STELLARIS 8 Leica DMi8 confocal microscope with a HC PL APO CS2 40x/1.25 GLYC oil immersion objective. Images were processed using Leica Application Suite X 3.7.4.23463 by Leica Microsystems.

2.7 Fluorescence-Activated Cell Scanning (FACS)

Flow cytometric analyses were conducted on a BD Accuri C6 from BD Biosciences. All obtained data were processed using FloJo™_v10.8.1_CL by BD Biosciences.

3. Syntheses

3.1 Cholesterol-CTA

3.1.1 Synthesis of Cholesteryl tosylate

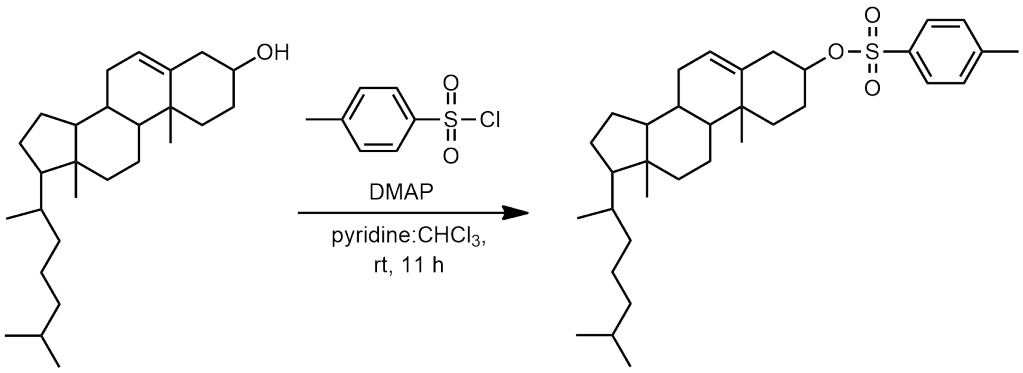

Figure S1: Synthesis of cholesteryl tosylate.

The synthesis was adapted from the literature and modified.[4] Cholesterol (5.0 g, 12.93 mmol, 1 eq) and 4-dimethylaminopyridine (DMAP) (0.158 g, 1.29 mmol, 0.1 eq) were dissolved in a mixture of dry $CHCl_3$ and pyridine (1:1) and cooled to 0 °C. Tosyl chloride (3.08 g, 16.16 mmol, 1.25 eq) dissolved in $CHCl_3$ (10 mL) was added dropwise *via* dropping funnel. After 1 h, the reaction mixture was allowed to warm to room temperature and stirred overnight, follow by extraction with brine (2x 50 mL), 1M HCl solution (2x 50 mL) and H_2O (2x 50 mL). The combined organic layers were dried over Na_2SO_4, filtrated, and concentrated *in vacuo*. The product was obtained as slightly yellow crystals (7.0 g, 100%).

^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 7.97 (d, J = 8.4 Hz, 2H, **A**), 7.33 (d, J = 8.3 Hz, 2H, **B**), 5.30 (dd, J = 5.3 Hz, 1H, **a**), 4.32 (tt, J = 11.0 Hz, 1H, **b**), 2.44 (s, 3H, **C**), 2.32-2.22 (m, 1H, **c**) 2.09-1.96 (m, 1H, **c'**), 1.86-1.74 (m, 3H, **d+e**), 1.72-1.29 (m, 12H, **f-l**), 1.28-0.97 (m, 10 H, **m-p**), 0.96 (s, 3H, **q**) 0.91 (d, 3H, **r**), 0.90-0.80 (m, 7H, **s-u**), 0.65 (s, 3H, **v**).

ESI-MS [m/z] = th.: 541.84 $[M+H]^+$, res.: 369.20 $[C_{27}H_{45}]^+$ (calc. 369.35), 448.20 $[C_{27}H_{45}\text{-}OSO_2]^+$ (calc. 448.31).

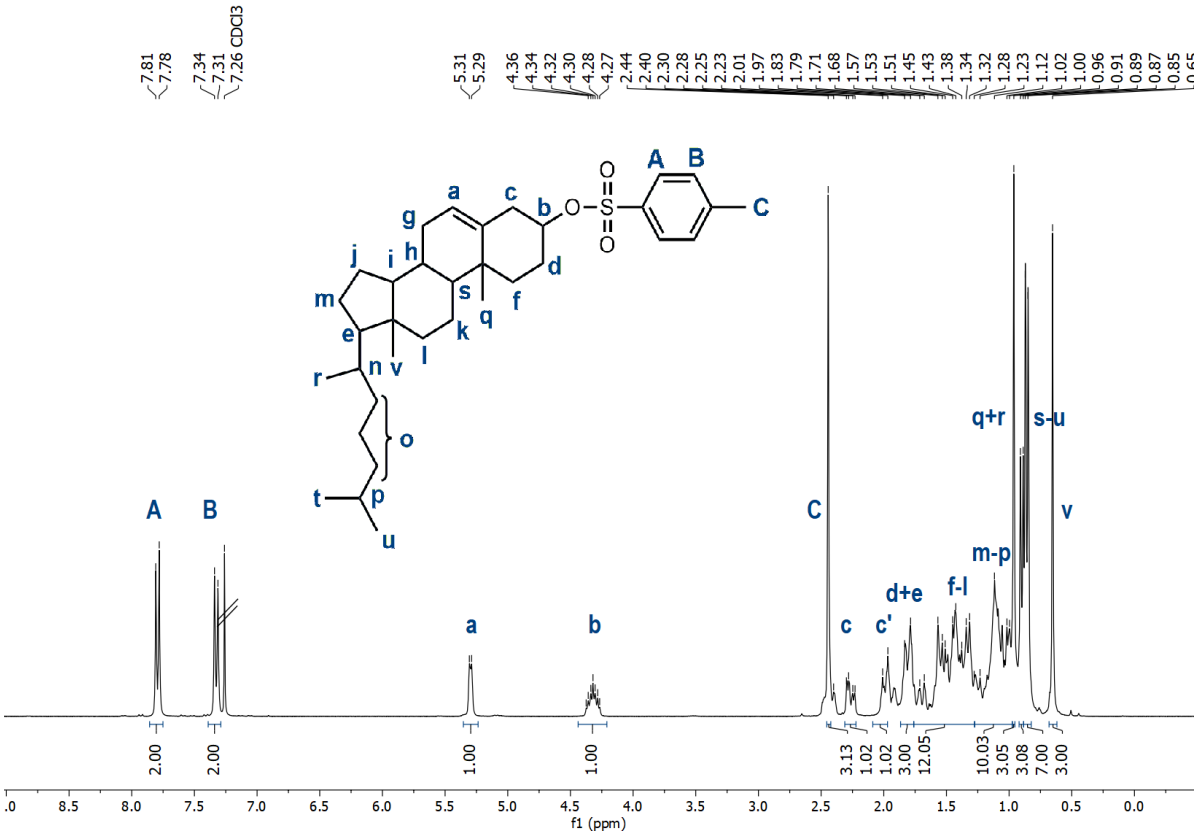

Figure S2: ^{1}H NMR spectrum (300 MHz) of cholesteryl tosylate in $CDCl_3$.

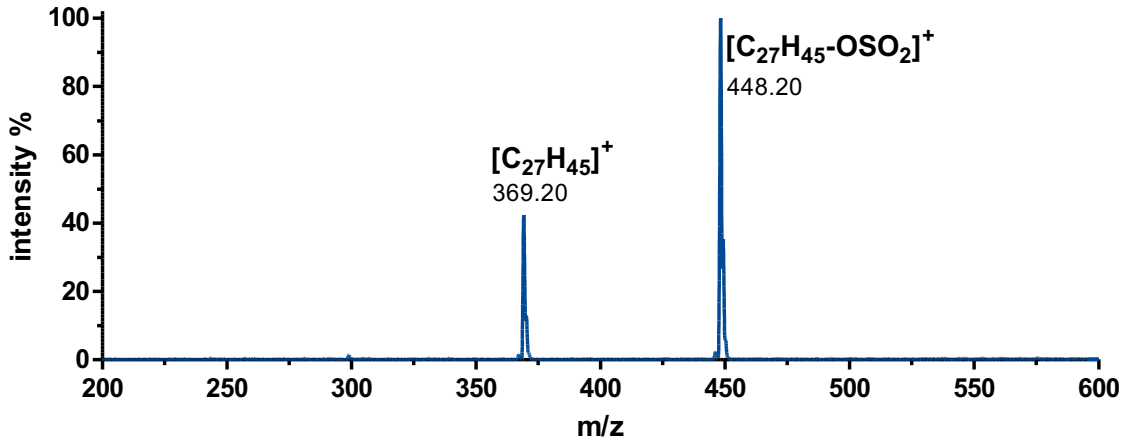

Figure S3: ESI-MS spectrum of cholesteryl tosylate in MeOH (positive ion mode).

3.1.2 Synthesis of Cholesteryl azide

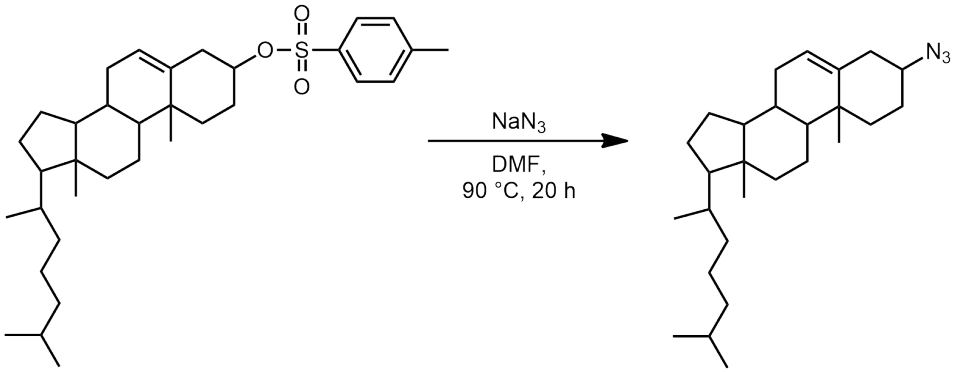

Figure S4: Synthesis of cholesteryl azide.

Cholesteryl azide was synthesized according to the literature with minor modification.[5,6] In an oven-dried two neck round bottom flask, cholesteryl tosylate (3.5 g, 6.47 mmol, 1 eq) was dissolved in dry DMF (50 mL) under nitrogen atmosphere. NaN_3 (0.841 mg, 12.94 mmol, 2 eq) was slowly added to the solution under nitrogen flow and stirred at 90 °C for 18 h. Thereafter, the reaction mixture was cooled to room temperature and quenched with water (100 mL). The organic phase was extracted with water (3x 30 mL) and the combined aqueous layer were washed with ethyl acetate (3x 30 mL). The organic solvent was dried over Na_2SO_4, filtrated, and evaporated under reduced pressure. The crude reaction product was purified by silica gel chromatography using cyclohexane and ethyl acetate (10:1) as eluent. Cholesteryl azide was isolated as colorless crystals (2.21 g, 89%).

^{1}H NMR (700 MHz, $CDCl_3$): δ (ppm) = 5.39 (s, 1H, **a**), 3.88 (s, 1H, **b**), 2.05-192 (m, 2H, **c**), 1.92-1.77 (m, 3H, **d+e**) 1.57-1.29 (m, 12H, **f-l**), 1.21-1.07 (m, 10H, **m-p**), 1.01 (s, 3H, **q**), 0.87 (d, *J* = 3.1 MHz, 3H, **r**), 0.87-0.85 (m, 7H, **s-u**), 0.68 (s, 3H, **v**).

^{13}C NMR (176 MHz, $CDCl_3$): δ (ppm) = 138.42 (**1**), 123.50 (**2**), 58.62 (**3**), 57.02 (**4**), 56.44 (**5**), 50.22 (**6**), 42.63 (**7**), 40.06 (**8**), 39.85 (**9**), 38.49 (**10**), 36.52 (**11**), 35.65 (**12**), 36.40 (**13**), 33.95 (**14**), 32.16 (**15**), 32.12 (**16**), 28.56 (**17**), 28.35 (**18**), 26.43 (**19**), 24.60 (**20**), 24.17 (**21**), 23.11 (**22**), 22.85 (**23**), 21.05 (**24**), 19.29 (**25**), 19.01 (**26**), 12.22 (**27**).

IR (ATR) ũ [cm^{-1}] = 840-790 (s, υ, CHR_3), 1390-1370 (m, δ, -CH_3), 1479-1430 (m, δ, -CH_2), 2160-2250 (m, υ, -N_3), 2960-2850 (s, υ, C-H).

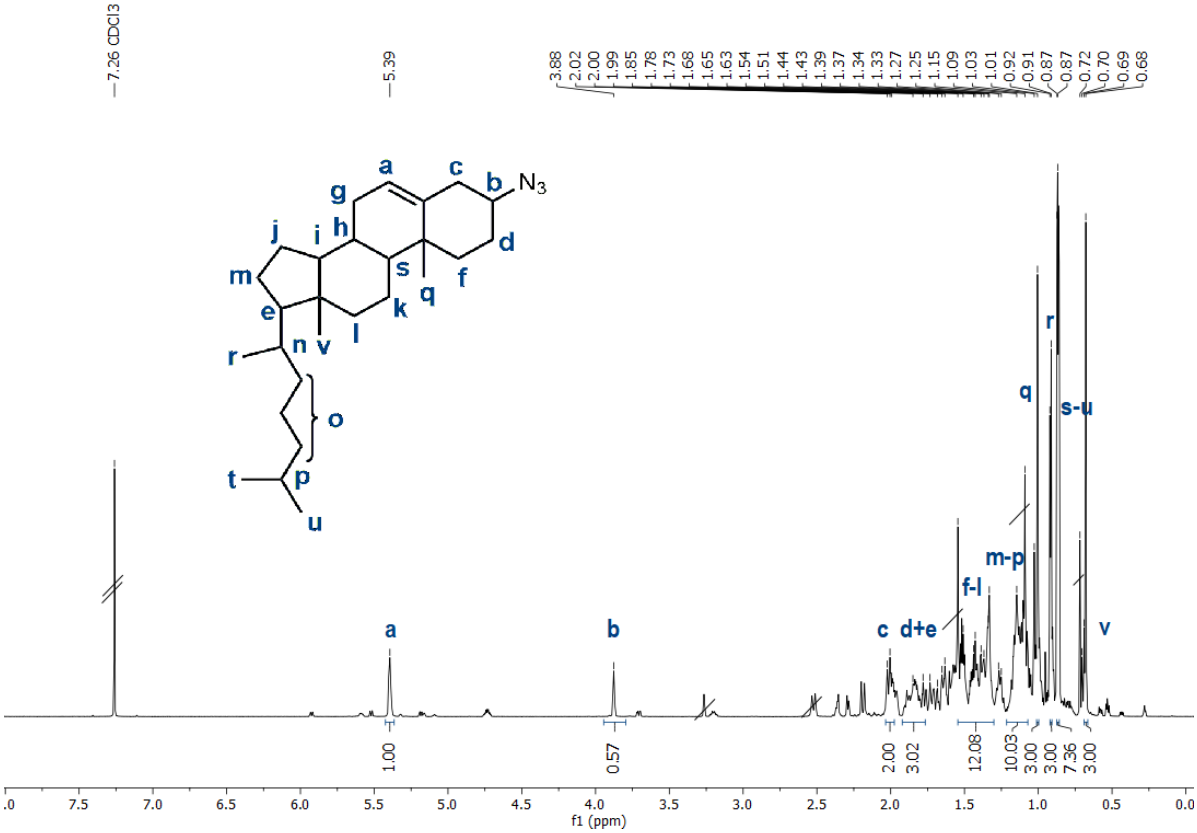

Figure S5: ^{1}H NMR spectrum (700 MHz) of cholesteryl azide in $CDCl_3$.

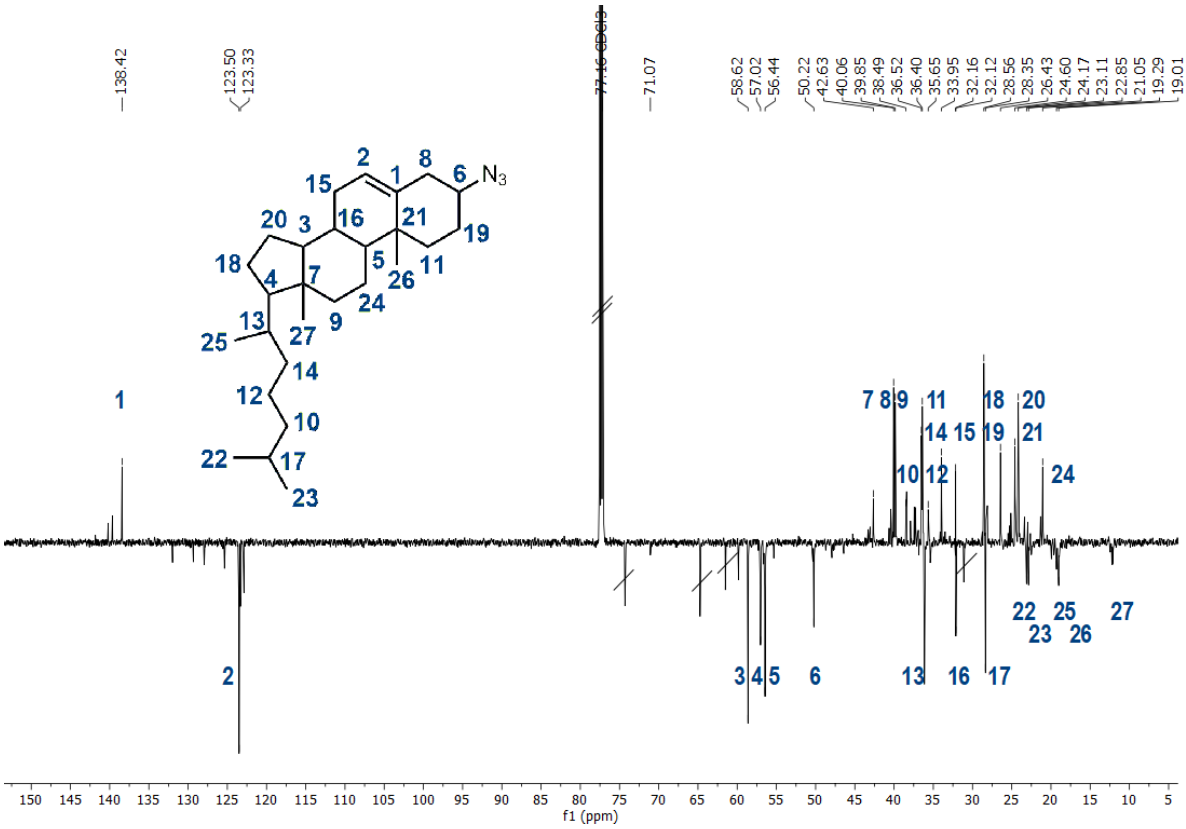

Figure S6: ^{13}C NMR spectrum (176 MHz) of cholesteryl azide in $CDCl_3$.

3.1.3 Synthesis of Cholesteryl amine

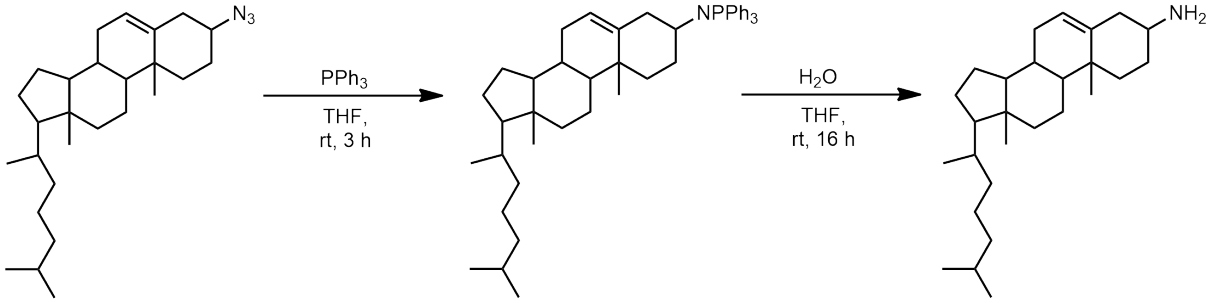

Figure S7: Synthesis of cholesteryl amine by Staudinger Reaction.

The synthesis of cholesteryl amine was conducted according to the literature and modified.[7] In a three neck round bottom flask, cholesteryl azide (2.0 g, 4.86 mmol, 1 eq) was dissolved in anhydrous THF (30 mL) under nitrogen atmosphere. To this solution triphenylphosphine (PPh_3) (6.37 g, 24.29 mmol, 5 eq) dissolved in dry THF (20 mL) was slowly added *via* dropping funnel. The reaction mixture was stirred at room temperature for 3 h. Afterwards, water (10 mL) was added, and the suspension was stirred for further 16 h. The reaction mixture was diluted with toluene (3x 200 mL) to remove THF and water by azeotropic evaporation *in vacuo*. The crude product was purified by column chromatography (DCM/MeOH 95:5 + 1% TEA) yielding cholesteryl amine as colorless powder (1.50 g, 80%).

^{1}H NMR (700 MHz, $CDCl_3$): δ (ppm) = 5.38 (s, 1H, **a**), 3.20 (d, *J* = 5.8 MHz, **b**), 2.61-2.51 (m, 1H, **c**), 2.13-2.07 (m, 1H, **c'**), 1.89-1.78 (m, 3H, **d+e**) 1.54-1.32 (m, 12H, **f-l**), 1.18-1.06 (m, 10H, **m-p**), 1.00 (s, 3H, **q**), 0.92 (d, *J* = 3.7 MHz, 3H, **r**), 0.86-0.85 (m, 7H, **s-u**), 0.67 (s, 3H, **v**).

^{13}C NMR (176 MHz, $CDCl_3$): δ (ppm) = 138.85 (**1**), 123.69 (**2**), 56.91 (**3**), 56.31 (**4**), 50.54 (**5**), 47.19 (**6**), 42.97 (**7**), 42.46 (**8**), 39.94 (**9**), 39.67 (**10**), 37.56 (**11**), 36.35 (**12**), 35.96 (**13**), 33.20 (**14**), 32.14 (**15**), 32.01 (**16**), 28.22 (**17**), 29.38 (**18**), 28.16 (**19**), 24.44 (**20**), 24.01 (**21**), 23.01 (**22**), 22.75 (**23**), 20.94 (**24**), 19.04 (**25**), 18.84 (**26**), 11.97 (**27**).

ESI-MS [m/z] = th.: 386.67 $[M+H]^+$, res.: 386.35 $[M+H]^+$, 369.09 $[C_{27}H_{45}]^+$ (calc. 369.35), 771.30 $[2M+H]^+$ (calc. 771.34).

IR (ATR) ũ [cm^{-1}] = 840-790 (s, υ, CHR_3), 1390-1370 (m, δ, $-CH_3$), 1479-1430 (m, δ, $-CH_2$), 1650-1560 (m, δ, $-NH_2$), 2960-2850 (s, υ, C-H), 3550-3050 (m, υ, N-H).

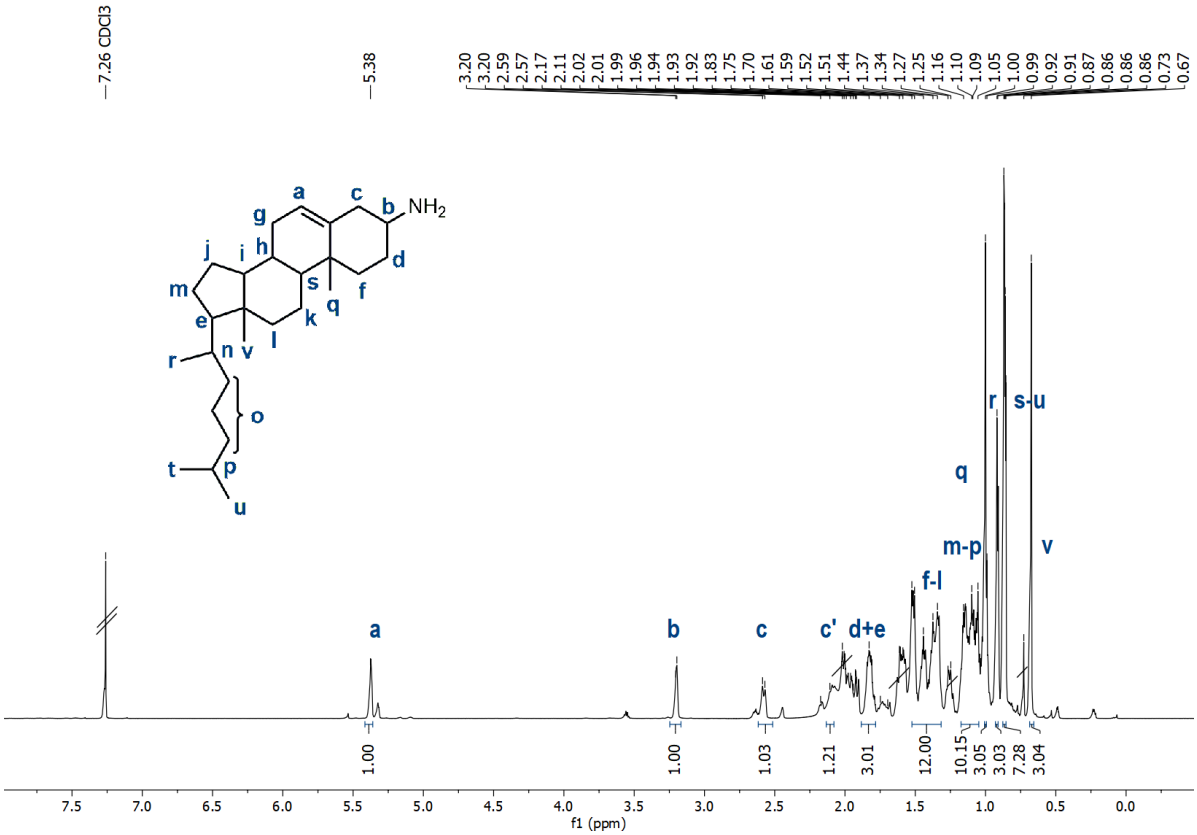

Figure S8: ^{1}H NMR spectrum (700 MHz) of cholesteryl amine in $CDCl_3$.

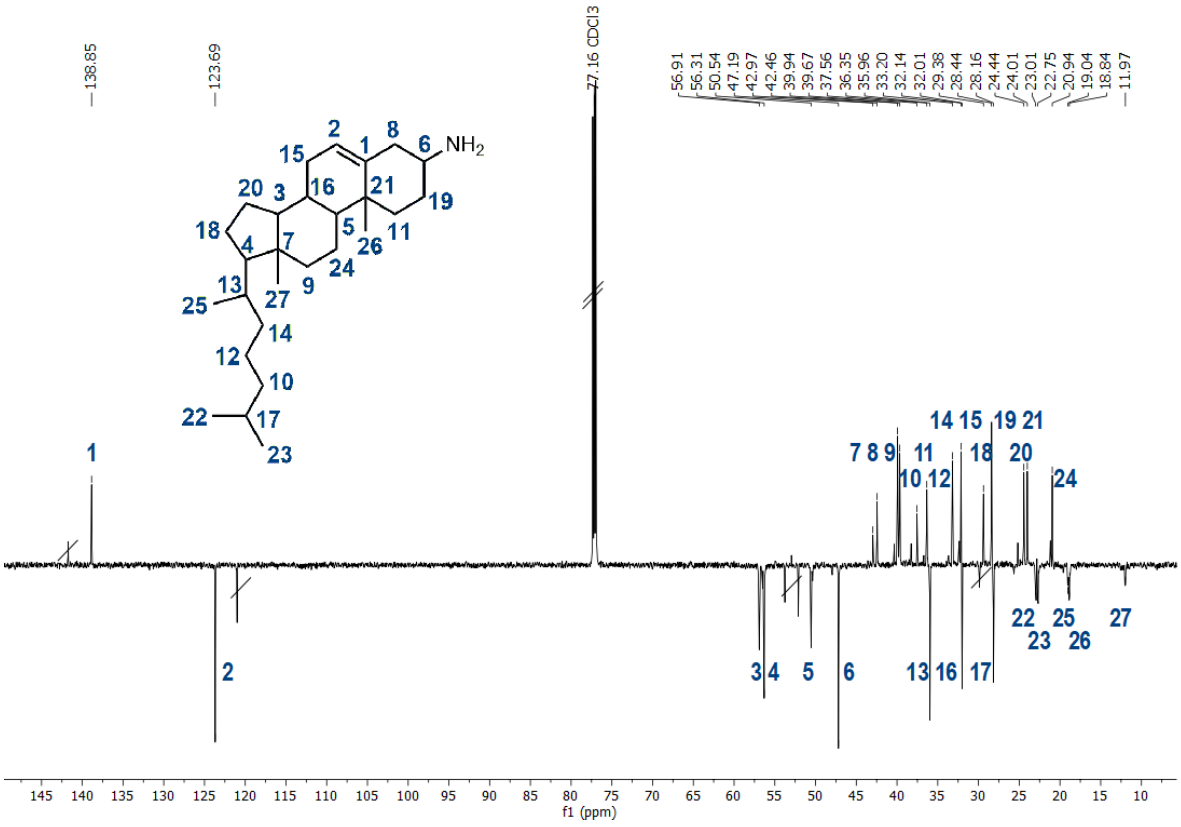

Figure S9: ^{13}C NMR spectrum (176 MHz) of cholesteryl amine in $CDCl_3$.

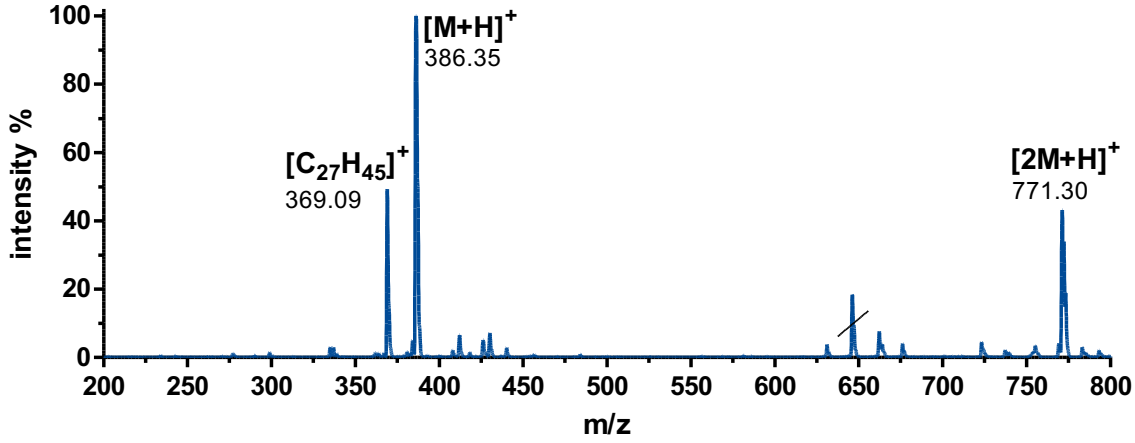

Figure S10: ESI-MS spectrum of cholesteryl amine in MeOH (positive ion mode).

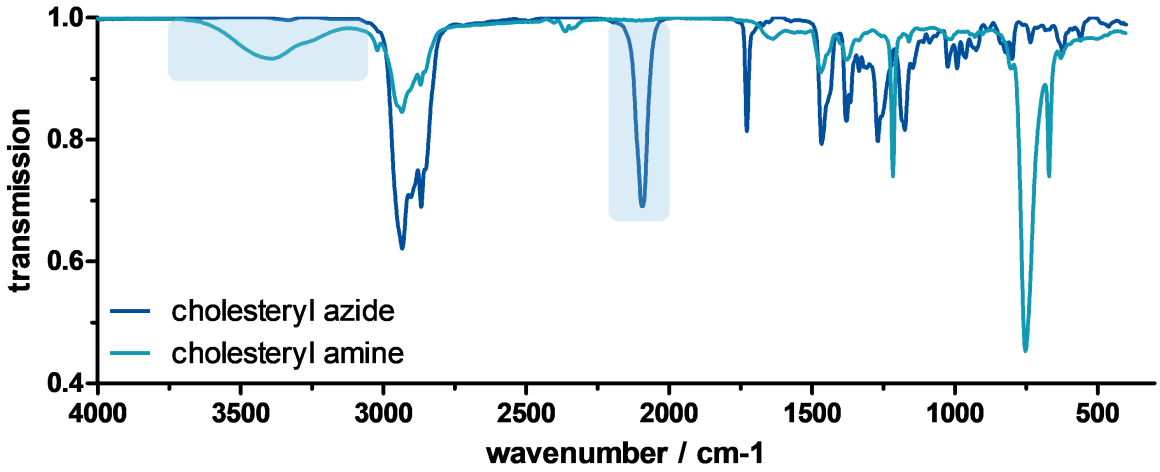

Figure S11: IR spectrum of cholesteryl azide (dark blue) and cholesteryl amine (light blue).

3.1.4 Synthesis of Pentafluorophenyl-trithiocarbonate Chain Transfer Agent

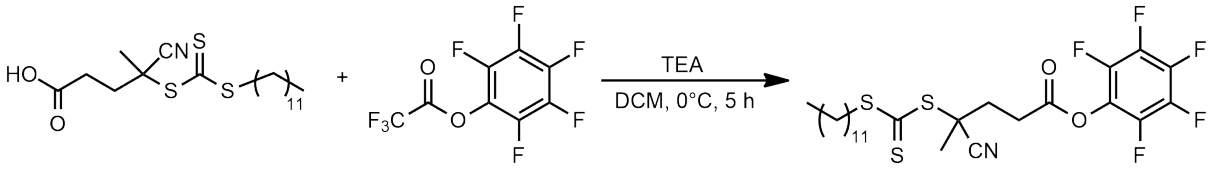

Figure S12: Synthesis of pentafluorophenyl-trithiocarbonate chain transfer agent (PFP-TTC-CTA).

Pentafluorophenyl-trithiocarbonate chain transfer agent (PFP-TTC-CTA) was synthesized by an adapted literature protocol.[8] 0.7 g 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]-pentanoic acid (TTC-CTA) (1.75 mmol, 1 eq) was weighed into a dry round bottom flask and dissolved in anhydrous DCM (20 mL) under nitrogen atmosphere. Triethylamine (TEA) (0.605 mL, 4.67 mmol, 2.5 eq) was added and the solution was cooled to 0 °C using an ice bath. Protected from light pentafluorophenyl trifluoroacetate (0.802 mL, 4.67 mmol, 2.5 eq) was

dropped to the reaction mixture *via* a syringe and stirred for 5 h at 0 °C. After complete conversion, the solution was diluted with DCM (15 mL) and the organic phase was extracted with water (3x 25 mL), dried over Na_2SO_4, filtrated, and evaporated *in vacuo*. The reaction product was purified *via* column chromatography (PE/EA 15:1). The product was isolated as yellow oil (0.75 g, 77 %).

^{1}H NMR (700 MHz, $CDCl_3$): δ (ppm) = 3.34 (t, *J* = 7.4 Hz, 2H, **a**), 3.05-2.98 (m, 2H, **b**), 2.68 (dt, *J* = 15.5 Hz, *J* = 8.0 Hz 1H, **c**), 2.53 (dt, *J* = 14.2 Hz, *J* = 7.6 Hz 1H, **c'**), 1.93 (s, 3H, **d**), 1.70 (quin, *J* = 7.1 Hz, 2H, **e**), 1.44-1.36 (m, 2H, **f**), 1.26 (s, 16H, **g**), 0.88 (t, *J* = 7.0 Hz, 3H, **h**).

^{19}F NMR (659 MHz, $CDCl_3$): δ (ppm) = -152.51 (d, *J* = 18 Hz, 2F, **a**), -157.29 (t, *J* = 21.6 Hz, 1F, **b**), -161.89 (d, *J* = 19.9 Hz, 2F, **c**).

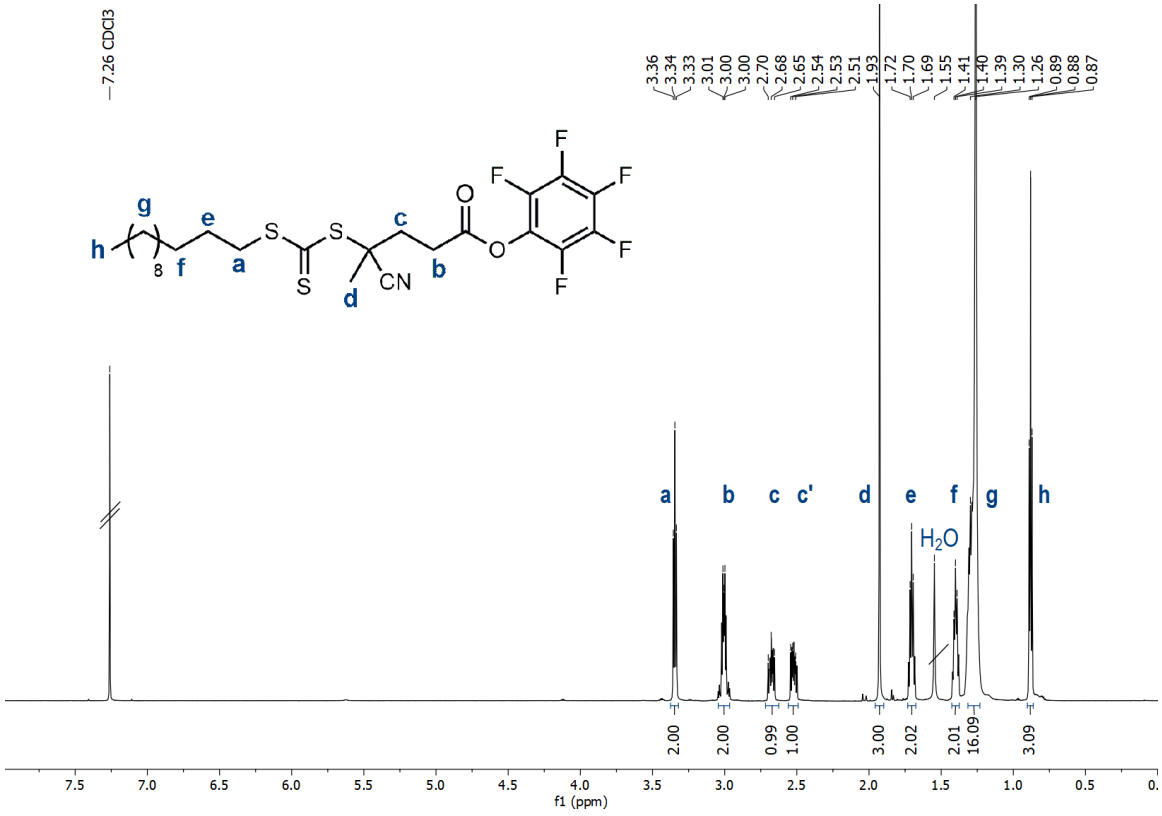

Figure S13: ^{1}H NMR spectrum (700 MHz) of pentafluorophenyl-trithiocarbonate chain transfer agent (PFP-TTC-CTA) in $CDCl_3$.

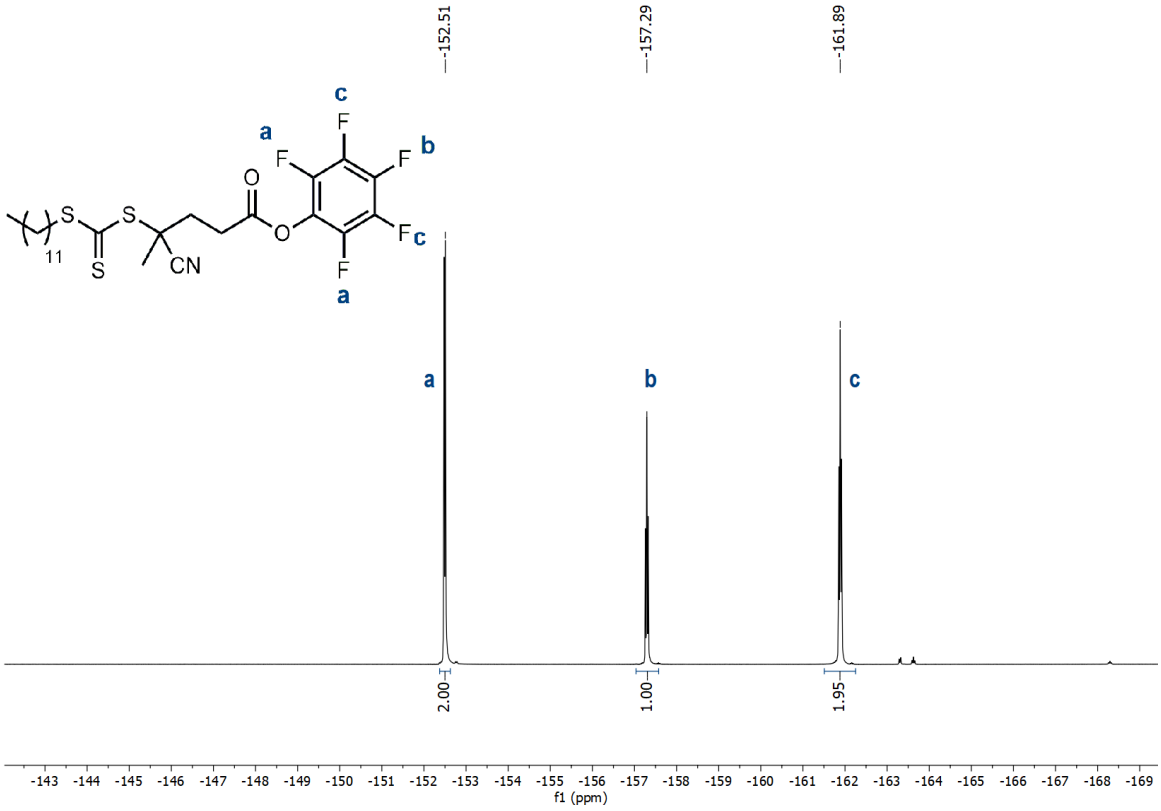

Figure S14: ^{19}F NMR spectrum (659 MHz) of pentafluorophenyl-trithiocarbonate chain transfer agent (PFP-TTC-CTA) in $CDCl_3$.

3.1.5 Synthesis of Cholesterol-trithiocarbonate Chain Transfer Agent (Chol-TTC-CTA)

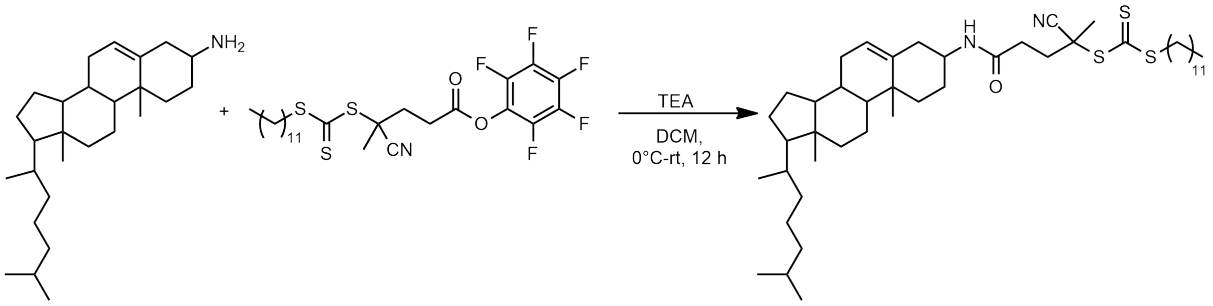

Figure S15: Synthesis of cholesterol-trithiocarbonate chain transfer agent (Chol-TTC-CTA).

The synthesis of cholesterol-trithiocarbonate chain transfer agent (Chol-TTC-CTA) was adapted from the literature and modified.[9] PFP-TTC-CTA (716 mg, 1.29 mmol, 2 eq) was weighed into a pre-dried two neck round bottom flask and dissolved in dry DCM (15 mL) under nitrogen atmosphere. The solution was cooled for 30 min using an ice bath and protected from light cholesteryl amine (250 mg, 0.648 mmol, 1 eq) dissolved in DCM (15 mL) and TEA (0.269 mL, 1.94, 3 eq) were dropwise added *via* dropping funnel. The reaction mixture was warmed to room temperature and stirred overnight. Then, the volatiles were evaporated under reduced pressure and the crude product was purified by silica gel chromatography using a gradient mixture of n-hexane and ethyl acetate (90:10, 80:20, 70:30 + 1% TEA). The cholesterol-TTC-CTA was obtained as yellow crystals (293 mg, 59 %).

^{1}H NMR (700 MHz, $CDCl_3$): δ (ppm) = 5.59 (d, *J* = 7.8 MHz, 1H, A), 5.41 (s, 1H, a), 4.13 (s, 1H, b), 3.33 (t, *J* = 7.5 MHz, 2H, B), 2.64-2.57 (m, 1H, c), 2.56-2.48 (m, 1H, C), 2.49-2.43 (m, 2H, D), 2.40-2.32 (m, 1H, C'), 2.07-2.00 (m, 1H, c'), 2.01 (s, 3H, E)1.87-1.72 (m, 3H, d+e), 1.74-1.64 (m, 2H, F), 1.58-1.31 (m, 12H, f-l), 1.30-1.27 (m, 2H, G), 1.26 (s, 16H, H), 1.22-1.06 (m, 10H, m-p), 1.02 (s, 3H, q), 0.92 (d, *J* = 6.6 MHz, 3H, r), 0.90-0.84 (m, 10H, I, s-u), 0.68 (s, 3H, v).

^{13}C NMR (176 MHz, $CDCl_3$): δ (ppm) = 217.47 (1), 169.32 (2), 138.78 (3), 124.14 (4), 119.46 (5), 56.85 (6), 56.34 (7), 50.52 (8), 46.96 (9), 46.01 (10), 42.46 (11), 39.85 (12), 39.65 (13), 37.52 (14), 37.21 (15), 36.33 (16), 35.96 (17), 34.75 (18), 34.28 (19), 32.26 (20), 32.05 (21), 31.95 (22), 29.76-29.09 (23-32), 28.95 (33), 28.38 (34), 28.15 (35), 26.14 (36), 25.23 (37), 24.41 (38), 24.03 (39), 23.00 (40), 22.66 (41), 20.91 (42), 19.01 (43), 18.96 (44), 14.30 (46), 12.02 (46).

MALDI-ToF [m/z] = 771.62 $[M]^+$ (calc. 771.32), 493.46 $[C_{33}H_{53}N_2O]^+$ (calc. 493.42).

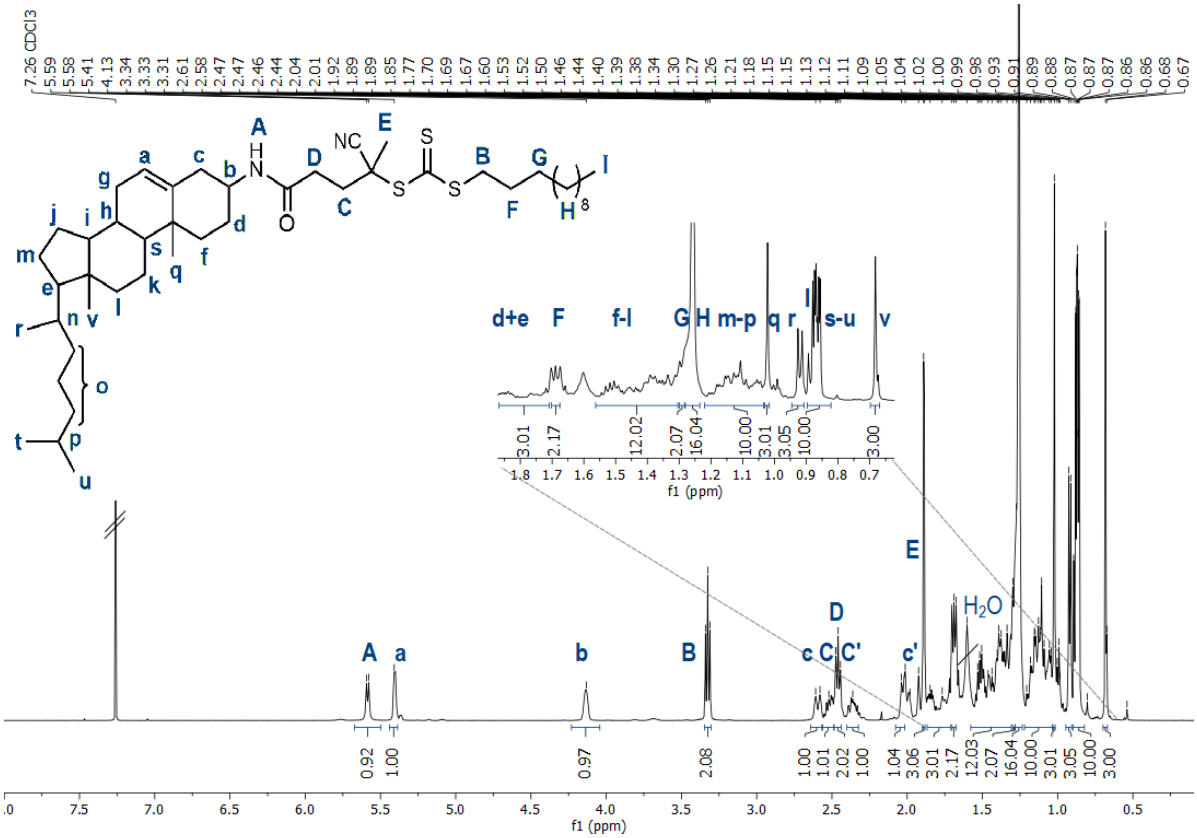

Figure S16: ^{1}H NMR spectrum (700 MHz) of Cholesterol-TTC-CTA in $CDCl_3$.

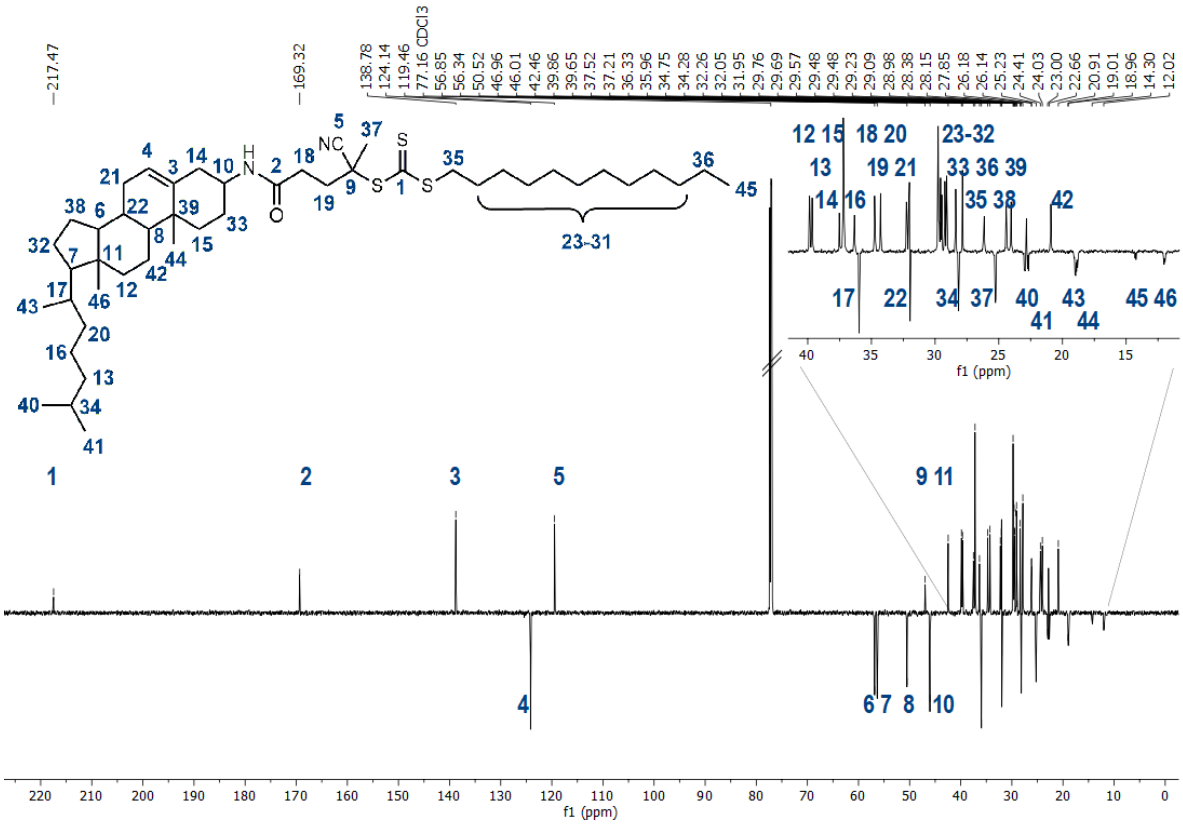

Figure S17: ^{13}C NMR spectrum (176 MHz) of Cholesterol-TTC-CTA in $CDCl_3$.

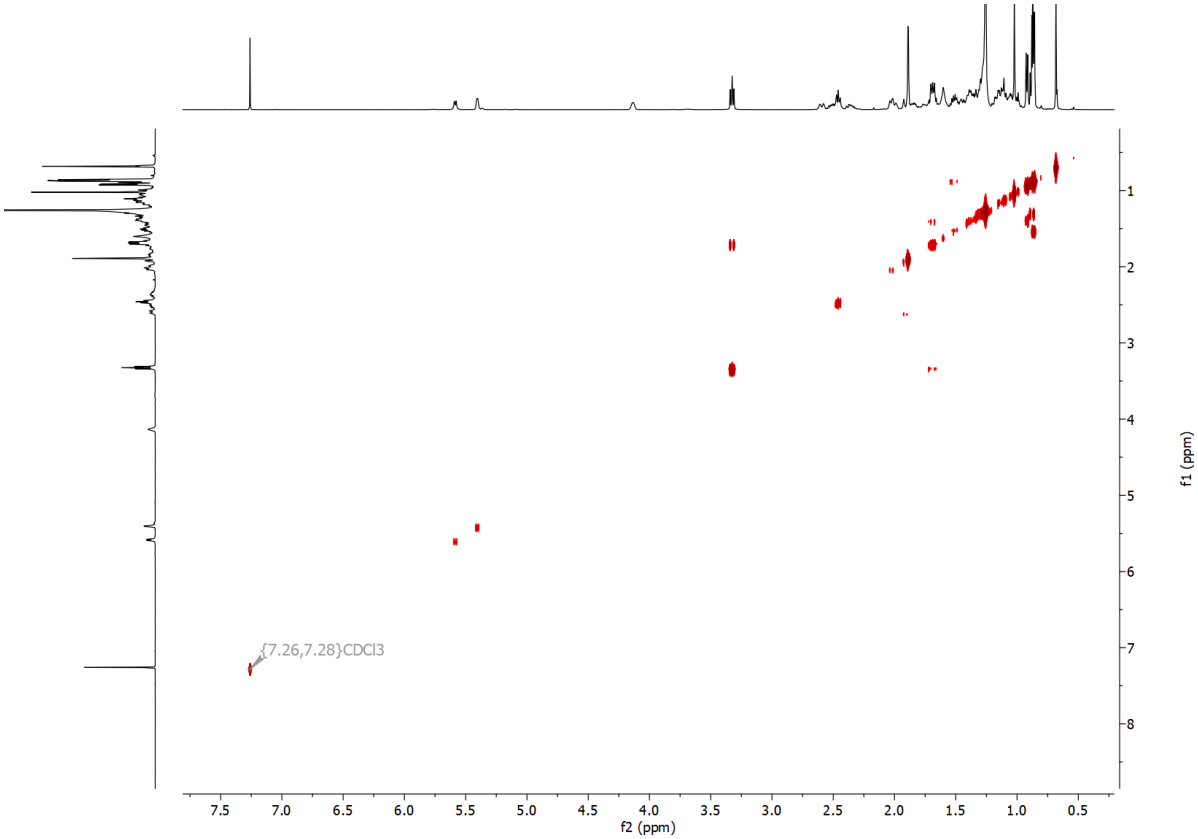

Figure S18: 2D COSY NMR spectrum of Cholesterol-TTC-CTA in $CDCl_3$.

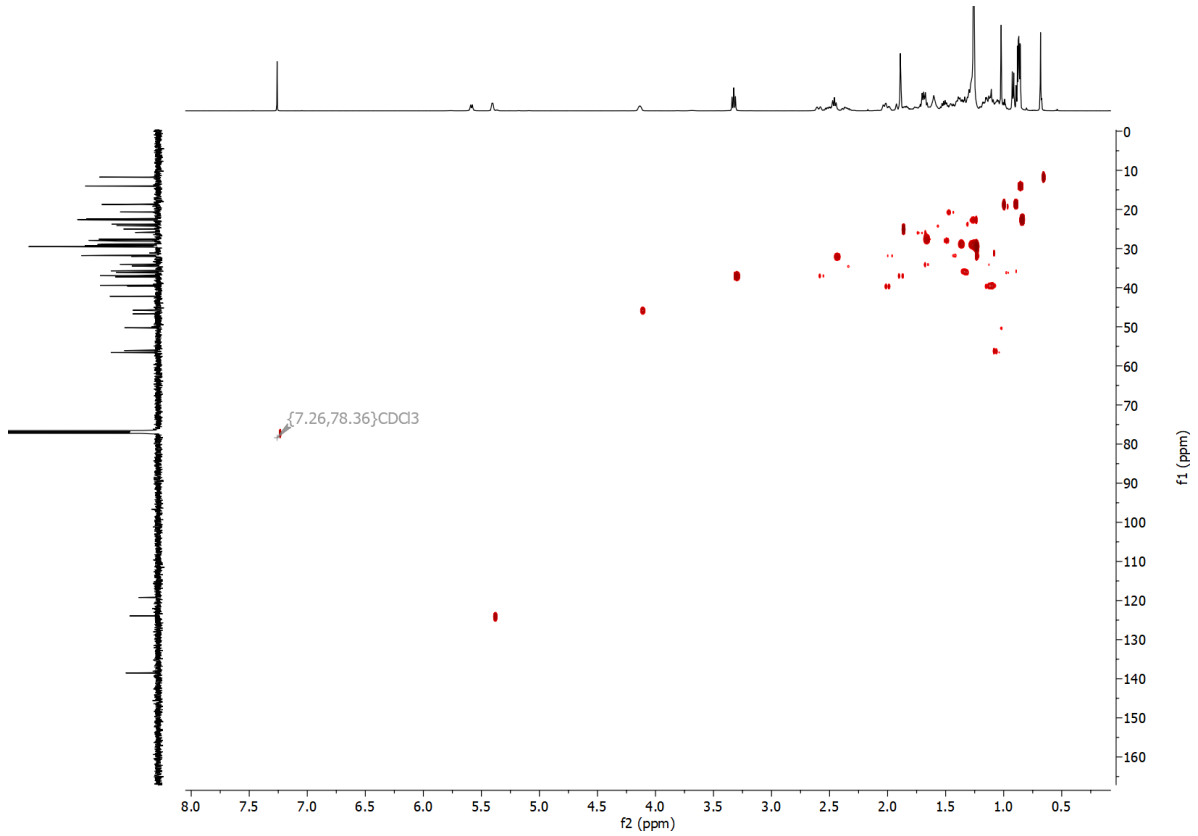

Figure S19: 2D HSQC NMR spectrum of Cholesterol-TTC-CTA in $CDCl_3$.

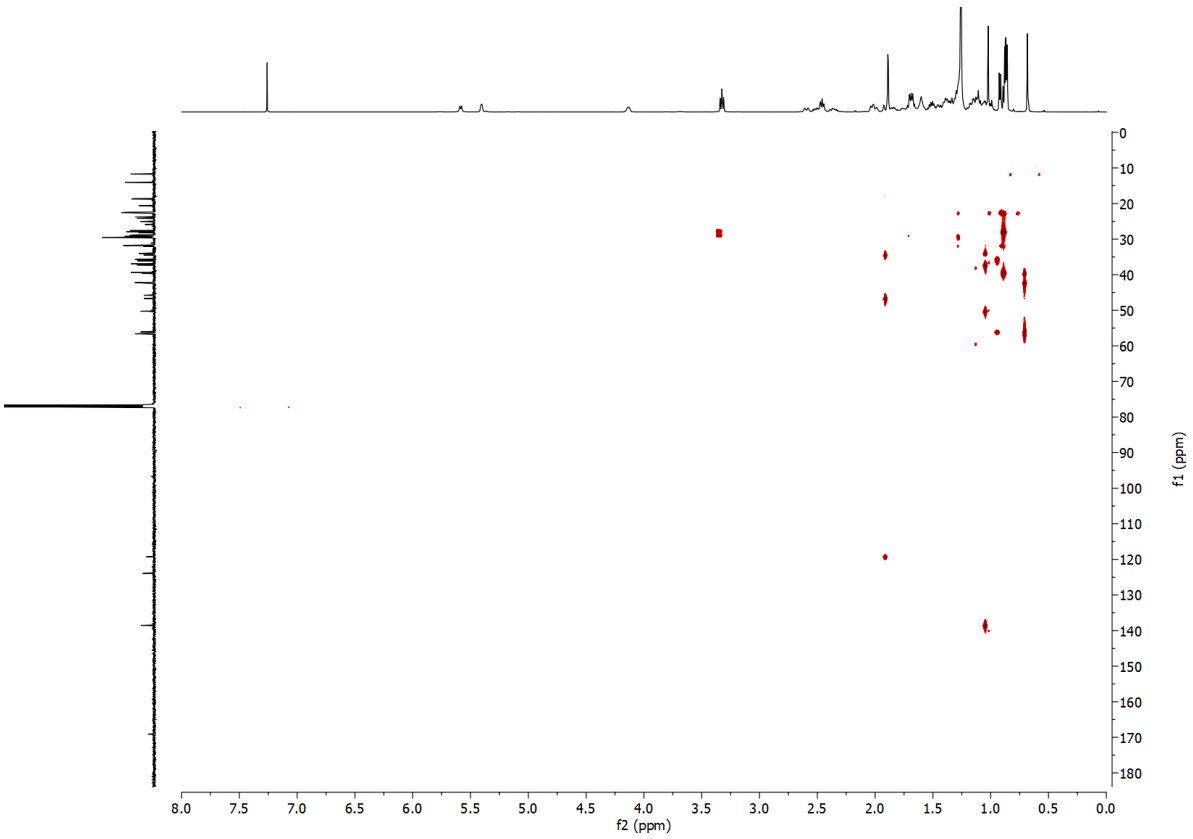

Figure S20: 2D HMBC NMR spectrum of Cholesterol-TTC-CTA in $CDCl_3$.

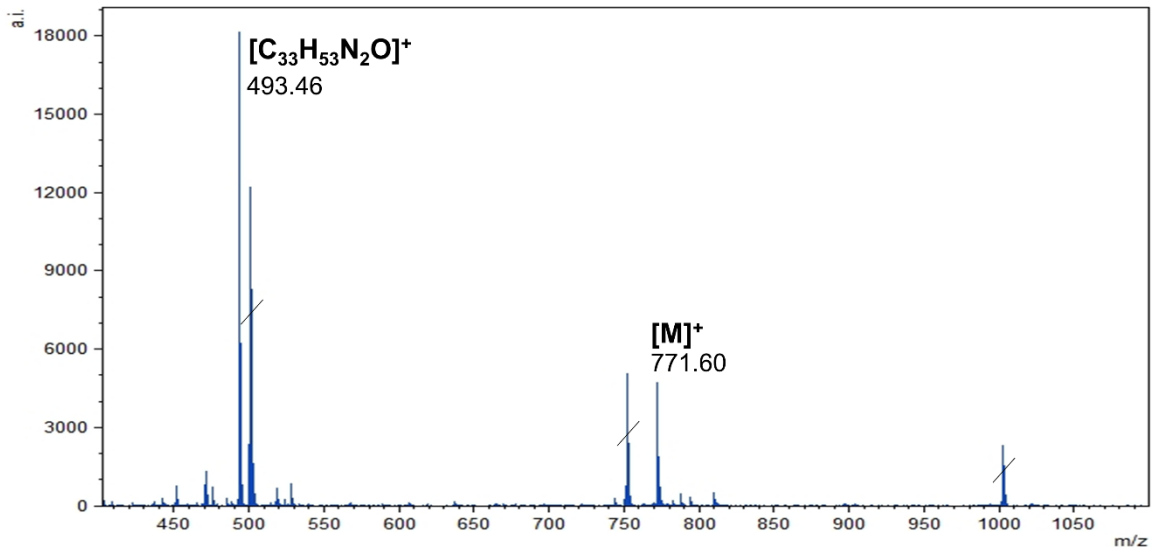

Figure S21: MALDI-ToF spectrum of Cholesterol-TTC-CTA in DCM.

3.2 Polymerization Procedure

3.2.1 Monomer Synthesis (PMMA-MA)

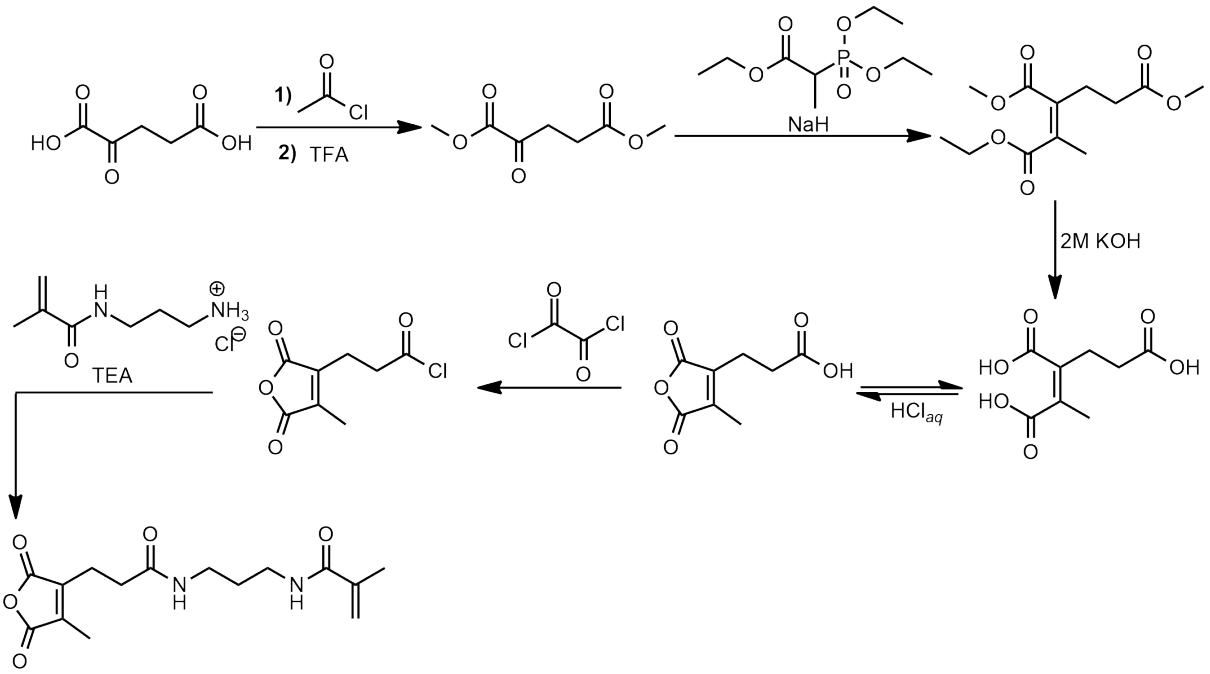

Figure S22: Synthesis scheme of the monomer N-(3-(3-(4-methyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanamido)-propyl)methacrylamide (PMMA-MA).

The monomer was synthesized as previously reported.[10]

^{1}H NMR (700 MHz, DMSO-d$_6$): δ (ppm) = 7.91 (s, 1H, **a**), 7.87 (s, 1H, **b**), 5.62 (s, 1H, **c**), 5.31 (s, 1H, **d**), 3.08 (q, *J* = 6.8 Hz, 2H, **e**), 3.02 (q, *J* = 6.8 Hz, 2H, **f**), 2.62 (t, *J* = 7.4 Hz, 2H, **g**), 2.35 (t, *J* = 7.4 Hz, 2H, **h**), 1.98 (s, 3H, **i**), 1.84 (s, 3H, **j**), 1.53 (quin, *J* = 8.4 Hz, 2H, **k**).

ESI-MS [m/z] = 309.10 $[M+H]^+$ (calc. 309.15), 331.15 $[M+Na]^+$ (calc. 331.13), 347.10 $[M+K]^+$ (calc. 347.10).

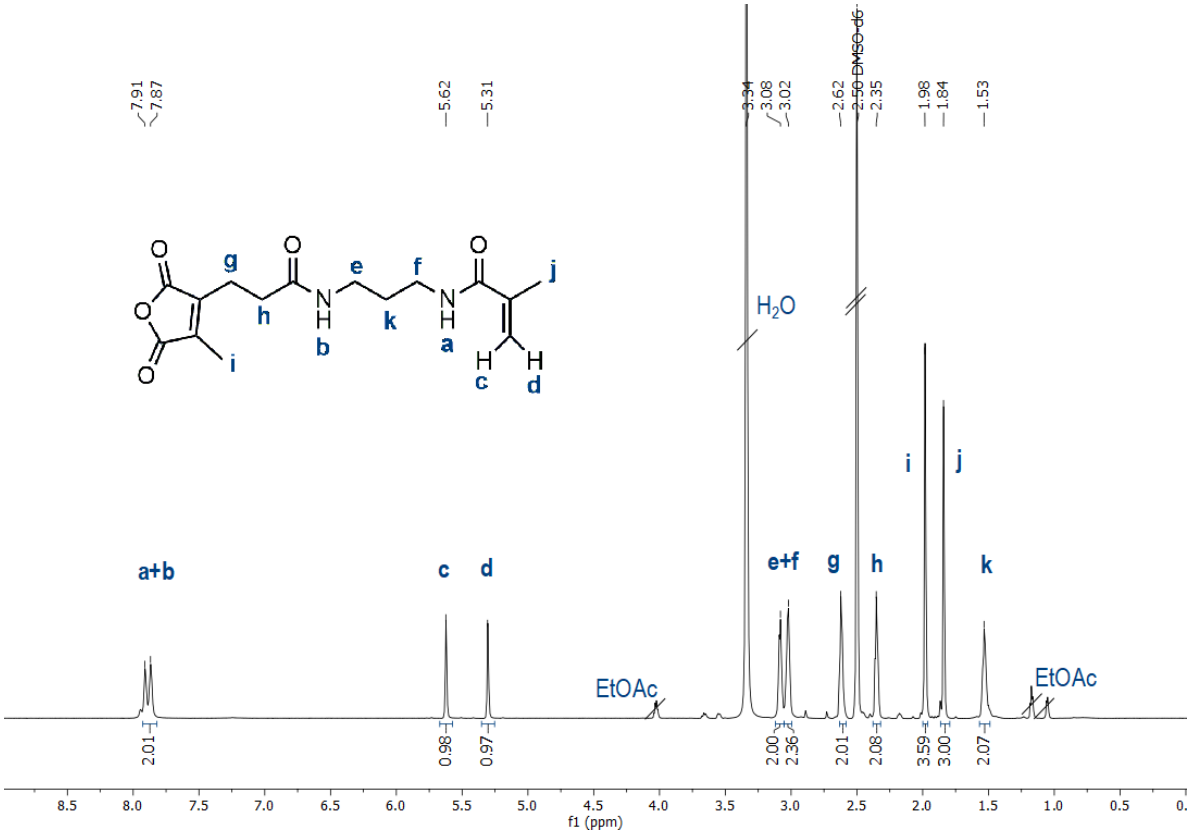

Figure S23: ^{1}H NMR spectrum (700 MHz) of the monomer N-(3-(3-(4-methyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanamido)-propyl)methacrylamide (PMMA-MA) in DMSO-d_6.

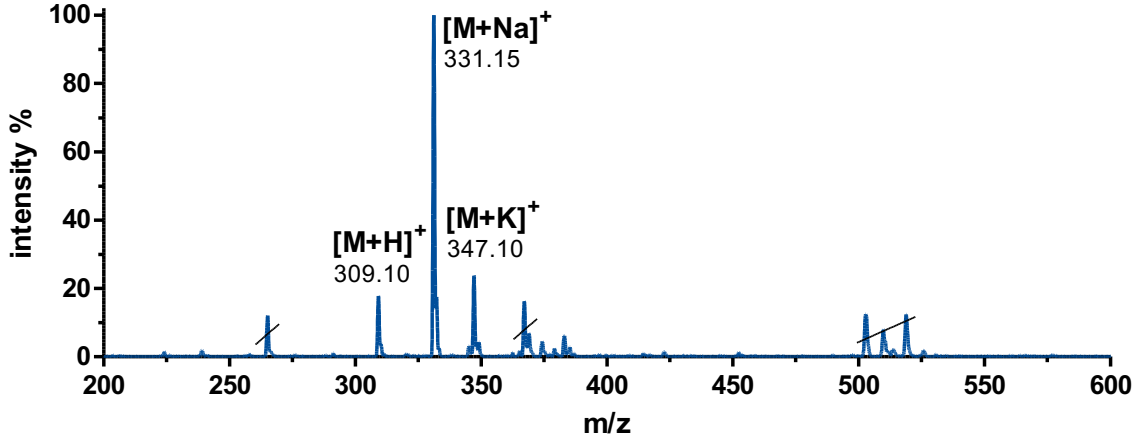

Figure S24: ESI-MS spectrum of N-(3-(3-(4-methyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanamido)-propyl)methacrylamide (PMMA-MA) in MeOH (positive ion mode).

3.2.2 Chol-Polymer Synthesis

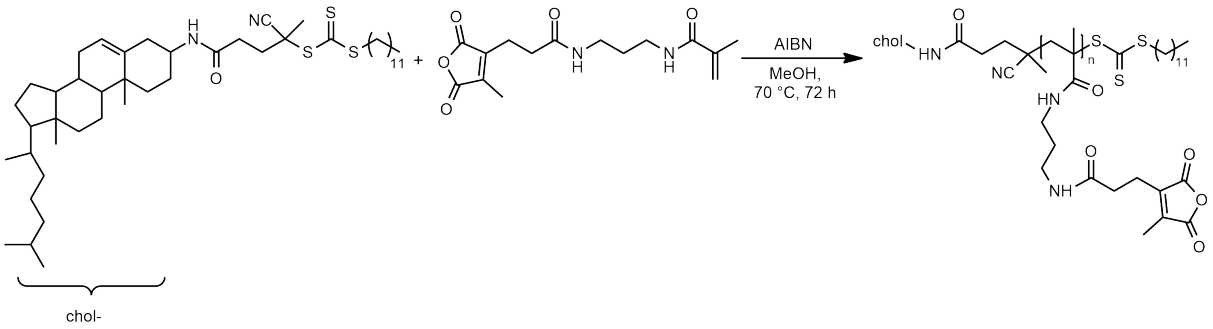

Figure S25: Synthesis of the chol-polymer with Chol-TTC-CTA and AIBN in MeOH for 72 h.

The chol-polymer was synthesized by RAFT polymerization according to the literature with minor modification.[10] Chol-TTC-CTA (7.48 mg, 9.7 µmol, 1 eq) and AIBN (0.48 mg, 2.9 µmol, 0.3 eq) were transferred into a pre-dried Schlenk flask and dissolved in 0.3 mL anhydrous methanol. PMMA-MA (150 mg, 486 µmol, 50 eq), dissolved in 0.3 mL dry methanol, was added to the mixture, followed by four freeze-pump-thaw cycles. The clear, yellow solution was stirred at 70 °C in the absence of light and oxygen. After a reaction time of 72 h, ^{1}H NMR analysis showed a monomer conversion of 60%. Threefold precipitation in cold diethyl ether (-20 °C) and drying *in vacuo* for 16 h yielded 75 mg of a pale-yellow solid (77%).

^{1}H NMR (700 MHz, DMSO-d$_6$): δ (ppm) = 7.95 (s, 1H, a), 7.81-7.14 (m, 1H, b), 5.23 (m, 1H, c), 4.09 (m, 1H, d), 3.75-3.45 (m, 2H, e), 3.08-2.94 (m, 4H, f+g), 2.62 (m, 2H, h), 2.36 (m, 2H, i), 1.99 (s, 3H, j), 1.84 (s, 3H, k), 1.57-1.44 (m, 4H, l+m), 1.33 (s, 16H, n), 1.06 (s, 3H, o), 1.00-0.83 (m, 12H, p-s), 0.82-0.68 (m, 3H, t), 0.65 (s, 3H, u).

SEC (HFIP): M_n = 2,500 g/mol, M_w = 3,100 g/mol, Đ = 1.23

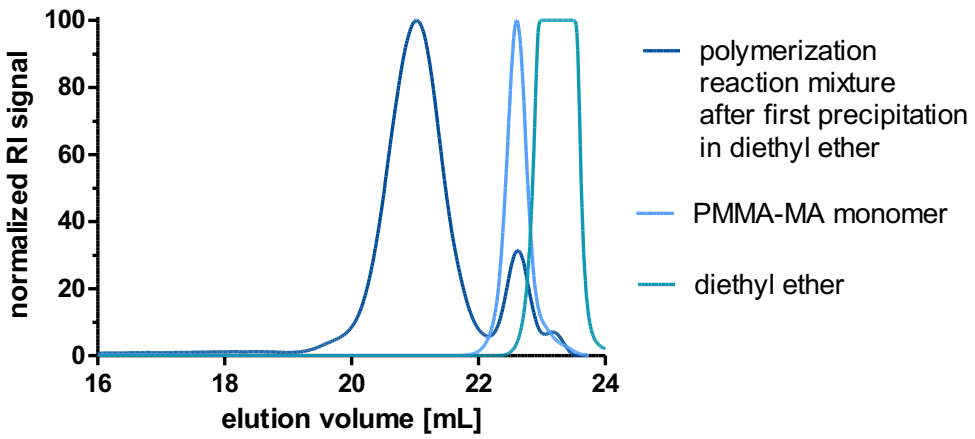

Figure S26: HFIP SEC trace of PMMA-MA RAFT polymerization reaction with Chol-TTC-CTA and AIBN in MeOH, followed by first precipitation in diethyl ether, as well as HFIP SEC traces of PMMA-MA monomer and diethyl ether. The minor peaks at 22.5 mL elution volume correspond to remaining traces of monomer and diethyl ether.

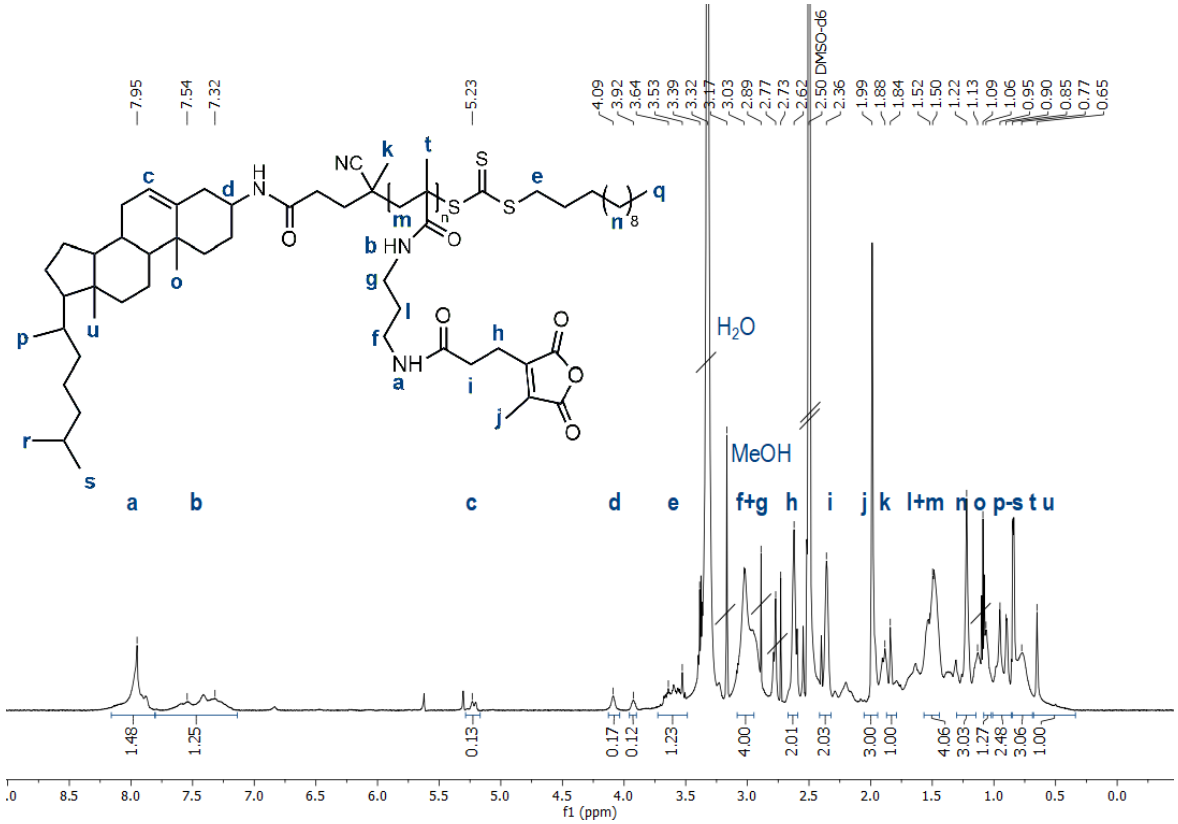

Figure S27: ^{1}H NMR spectrum (700 MHz) of the chol-polymer synthesized by RAFT polymerization in DMSO-d_6.

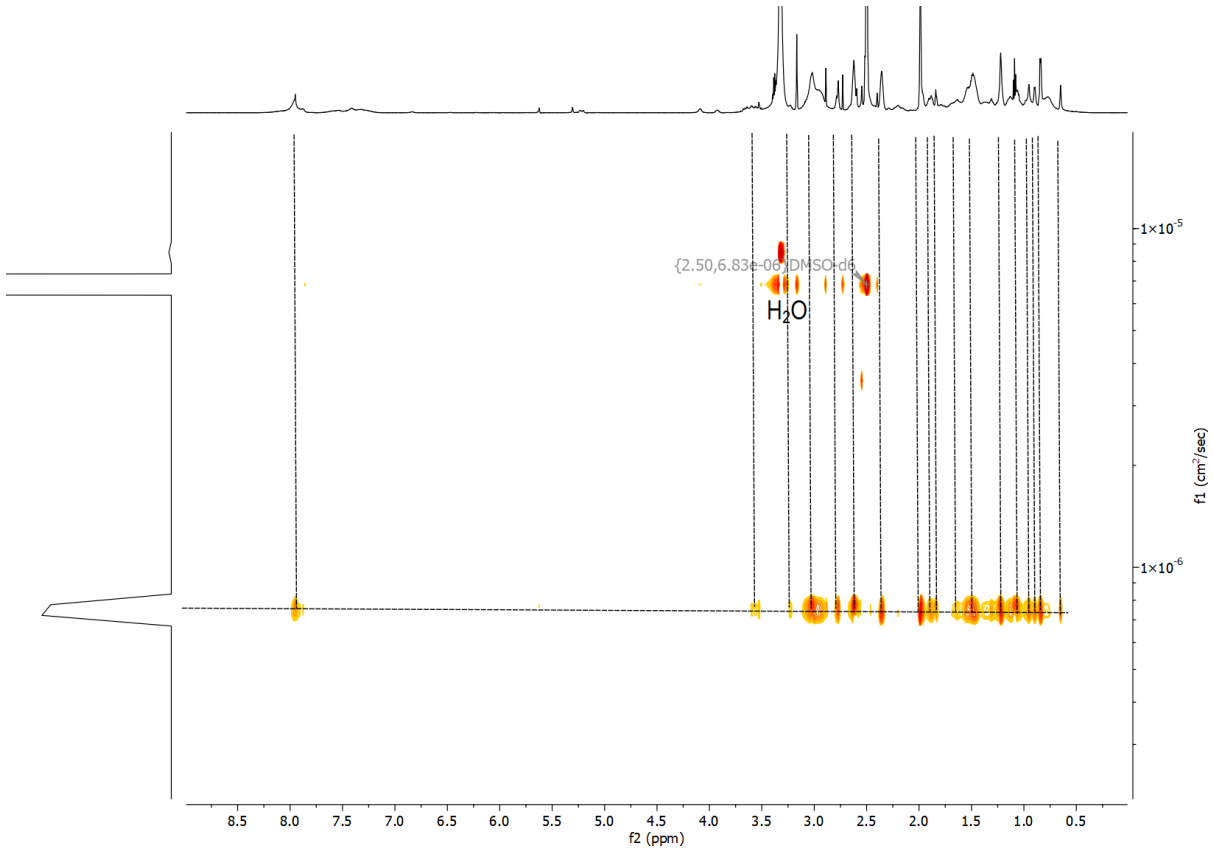

Figure S28: DOSY spectrum (700 MHz) of the chol-polymer synthesized by RAFT polymerization in DMSO-d_6.

Homopolymer Synthesis

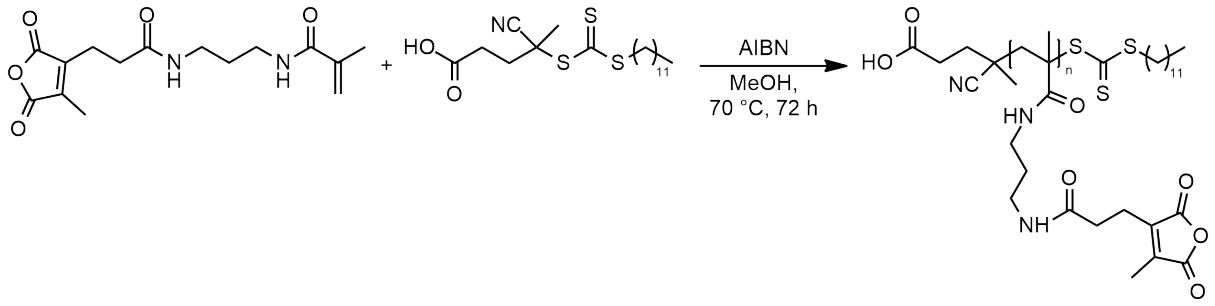

Figure S29: Synthesis of the polymer with TTC-CTA and AIBN in MeOH for 72 h.

The homopolymer was synthesized analogously to the chol-polymer procedure with 4-cyano-4-(((dodecylthio)carbonothioyl)thio)pentanoic acid (TTC-CTA) as chain transfer agent. AIBN (0.48 mg, 2.9 µmol, 0.3 eq) and TTC-CTA (3.92 mg, 9.7 µmol, 1 eq) were weighed in a Schlenk flask and dissolved in 0.3 mL dry methanol. Then, PMMA-MA (150 mg, 486 µmol, 50 eq), dissolved in 0.3 mL methanol, was transferred to the flask and the reaction mixture was degassed by four freeze-pump-thaw cycles. After heating at 70 °C for 72 h the conversion was determined by ^{1}H NMR analysis (69% conversion) and the polymer was precipitated in cold diethyl ether. The polymer was isolated as colorless solid (79 mg, 67%).

^{1}H NMR (700 MHz, DMSO-d_6): δ (ppm) = 7.95 (s, 1H, a), 7.72-7.18 (m, 1H, b), 3.02-2.95 (m, 4H, c+d), 2.62 (m, 2H, e), 2.36 (m, 2H, f), 1.99 (s, 3H, g), 1.69-1.41 (m, 4H, h+i), 1.22 (s, 2H, j) 1.04-0.53 (m, 3H, k).

SEC (HFIP): M_n = 2,300 g/mol, M_w = 2,900 g/mol, Đ = 1.20

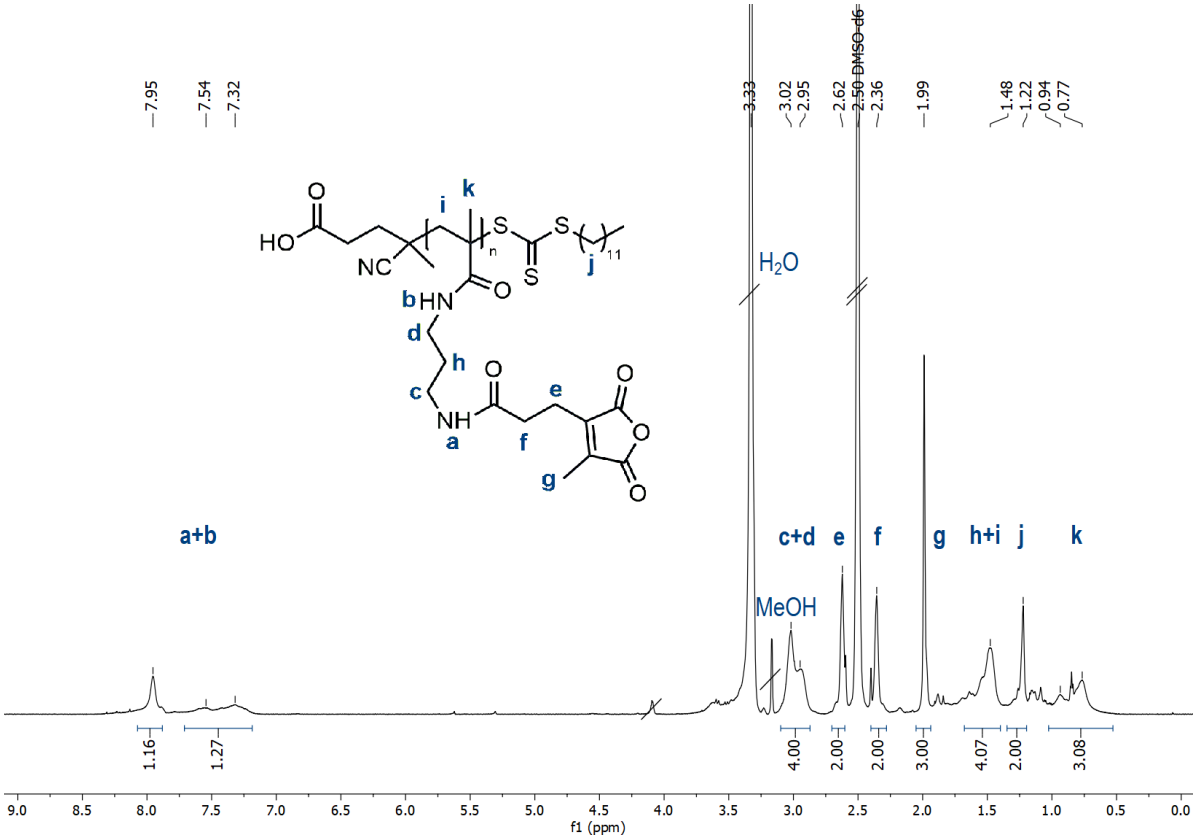

Figure S30: ^{1}H NMR spectrum (700 MHz) of the polymer synthesized under RAFT conditions in DMSO-d_6.

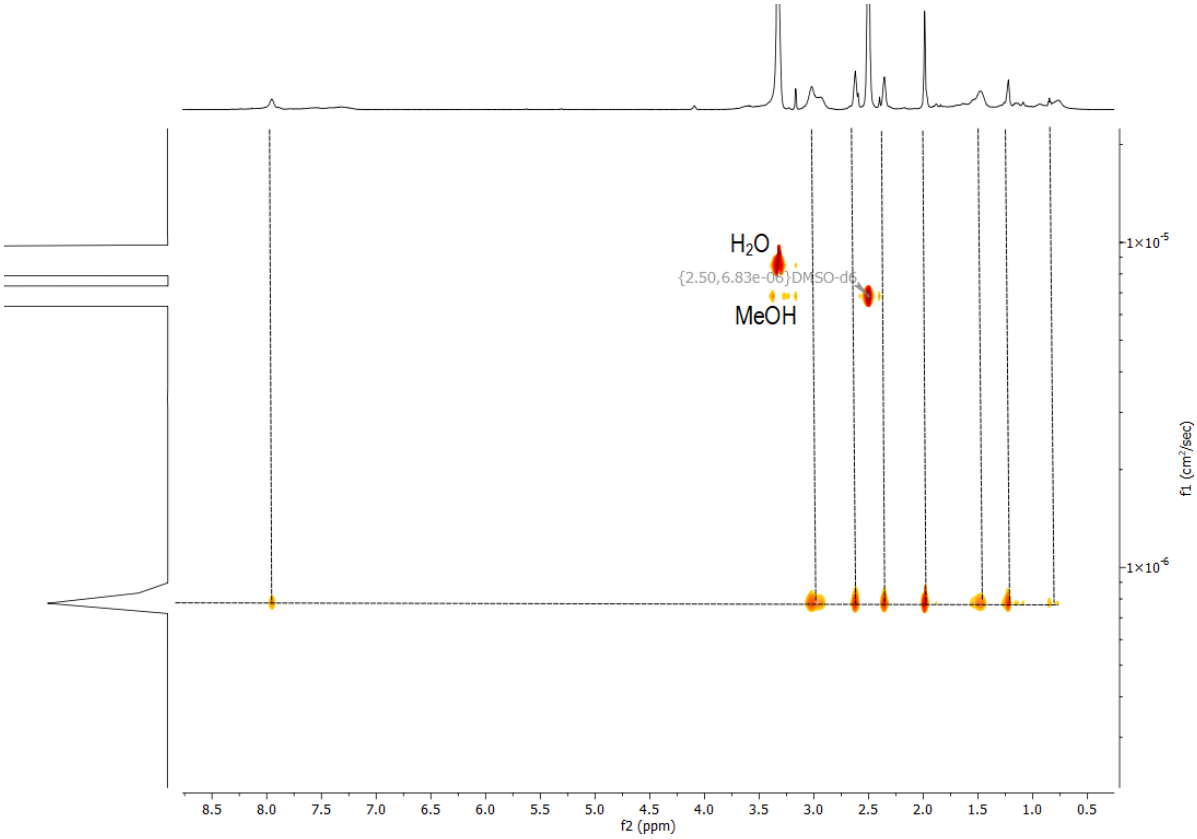

Figure S31: DOSY spectrum (700 MHz) of the polymer synthesized under RAFT conditions in DMSO-d_6.

3.3 pH-Reversible or Irreversible Polymer Modification by Amidation with Primary and Secondary Amines

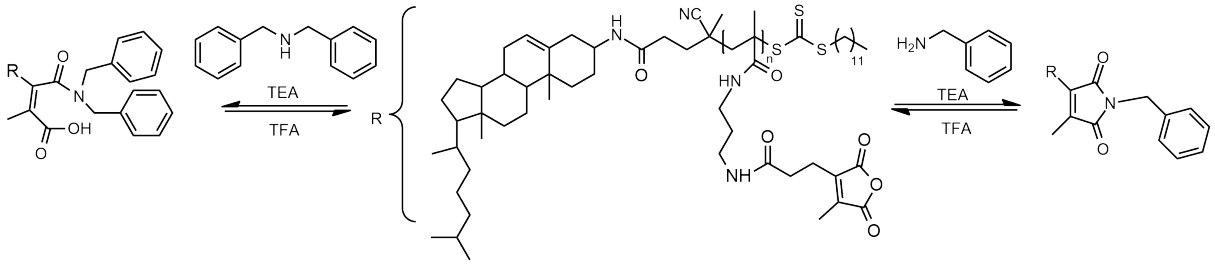

Figure S32: Schematic pH-sensitive reaction of chol-polymer chol-p(PMMA-MA)$_{30}$ with dibenzylamine or benzylamine.

The ability for pH-reversible amine conjugation was analyzed by reacting primary (benzylamine) or secondary (dibenzylamine) amines with the chol-polymer. In a Schlenk tube equipped with a stirring bar 10 mg chol-polymer (1.0 µmol/29.9 µmol reactive anhydride groups, 1 eq) was dissolved in DMSO under nitrogen atmosphere. Triethylamine (TEA) (20.79 µL, 150 µmol, 5 eq) and the corresponding amines, such as dibenzylamine (17.25 µL, 89.7 µmol, 3 eq) or benzylamine (9.81 µL, 89.7 µmol, 3 eq) were dropped to the reaction mixture. After 16 h heating at 50 °C the product was precipitated three times in diethyl ether and dried under reduced pressure overnight. The modified polymers were isolated as pale-yellow solid (14 mg, 88%) and analyzed by ^{1}H NMR and DOSY measurement.

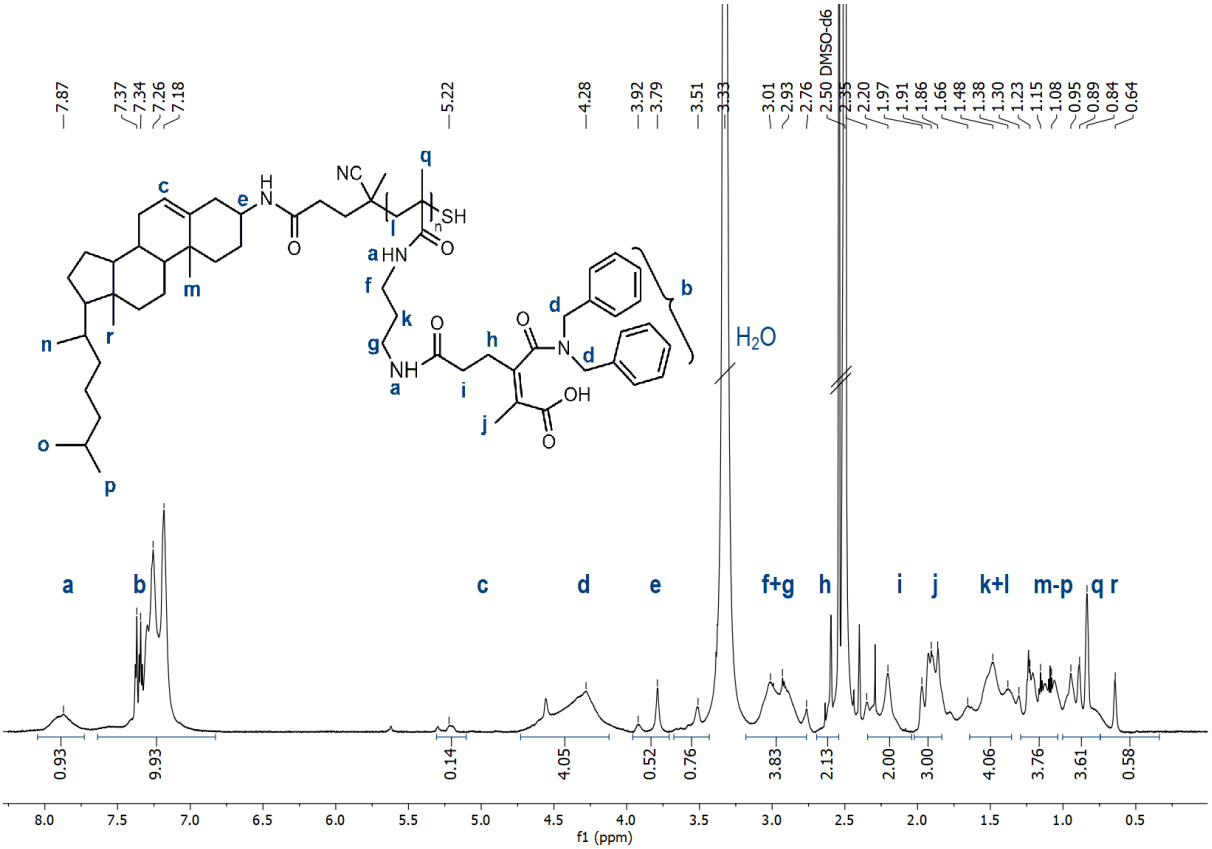

Figure S33: ^{1}H NMR spectrum (700 MHz) of the chol-polymer after modification with dibenzylamine under neutral in DMSO-d_6.

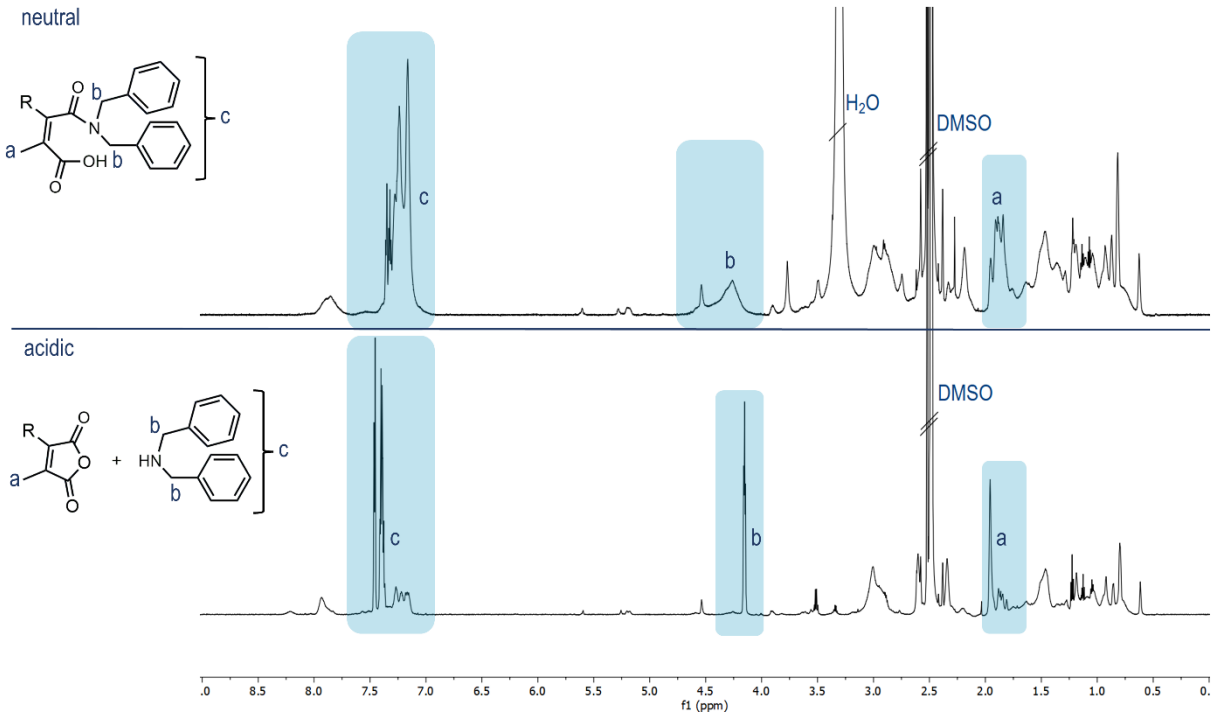

Figure S34: ^{1}H NMR spectra (700 MHz) of the chol-polymer after modification with dibenzylamine under neutral and acidic conditions in DMSO-d_6.

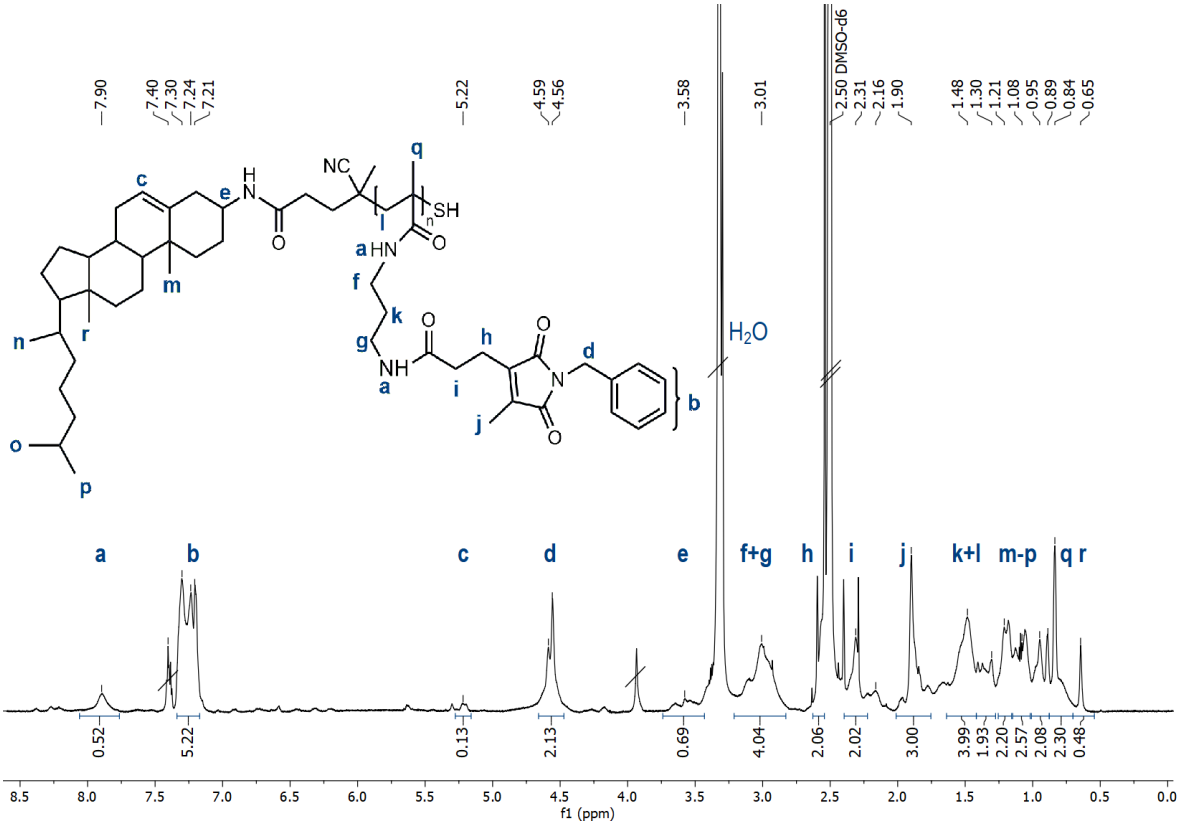

Figure S35: ^{1}H NMR spectrum (700 MHz) of the chol-polymer after modification with benzylamine under neutral in DMSO-d_6.

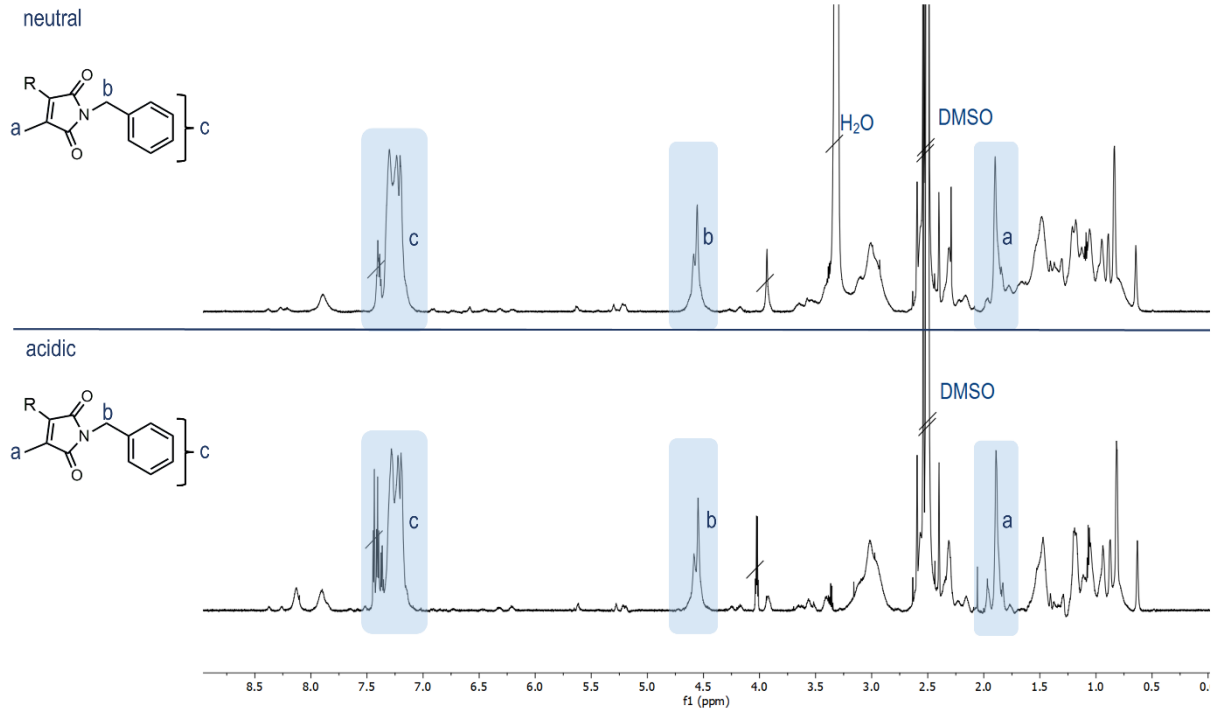

Figure S36: 1H NMR spectra (700 MHz) of the chol-polymer after modification with benzylamine under neutral and acidic conditions in DMSO-d_6.

3.4 Formulation of Dye-Labeled and PEG-Conjugated Polymers by Amidation

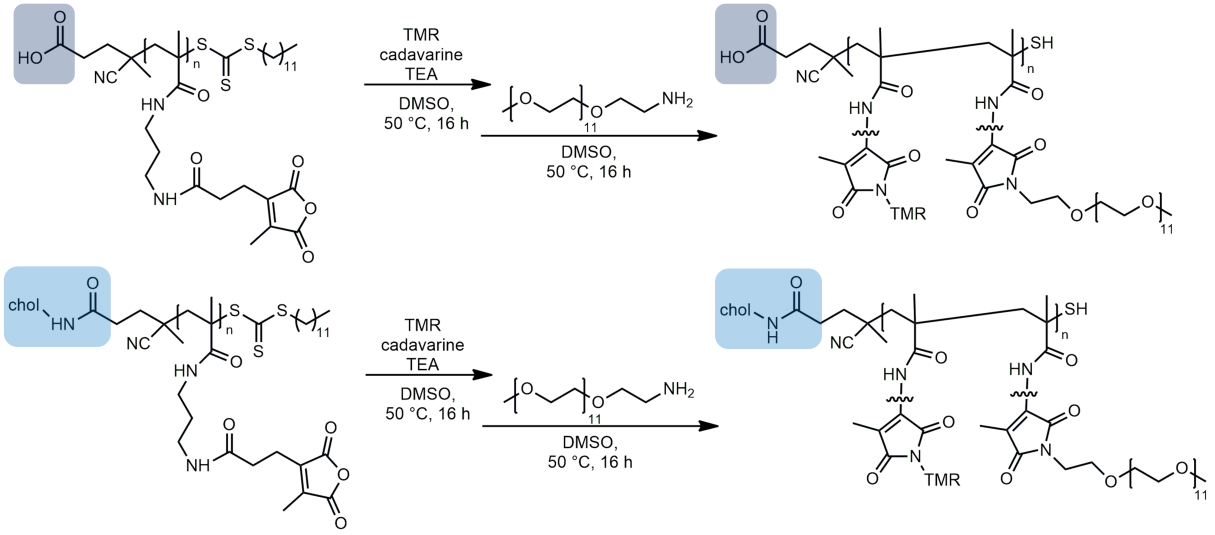

Figure S37: Schematic reaction of the homopolymer p(PMMA-MA)$_{38}$ or the chol-polymer chol-p(PMMA-MA)$_{30}$ with TMR cadaverine and mPEG$_{11}$-amine.

15 mg chol-polymer (1.50 µmol/45.0 µmol reactive anhydride groups, 1 eq) or homopolymer (1.24 µmol/47.0 µmol reactive anhydride groups, 1 eq) were dissolved in 0.5 mL anhydrous DMSO under nitrogen atmosphere. Next, TEA (31.15 µL, 225 µmol, 5 eq) / (32.57 µL, 235 µmol, 5 eq) and tetramethylrhodamine cadaverine (46.31 µL of a 5 mg/mL stock solution in DMSO, 0.45 µmol, 0.01 eq) / (48.84 µL of a 5 mg/mL stock solution in DMSO, 0.47 µmol, 0.01 eq) were added to the solutions. The reaction mixtures were stirred at 50 °C overnight. After dye conjugation, the chol-polymer and the homopolymer were reacted with mPEG$_{11}$-amine (M_n: 0.75 kDa) (1.04 mL of a 100 mg/mL stock solution in DMSO, 138 µmol, 3 eq)/ (1.06 mL, 100 mg/mL in DMSO, 141 µmol, 3 eq) and stirred for further 16 h at 50 °C. Finally, the solutions were three times precipitated in an excess of diethyl ether, centrifuged (4000 rpm, 20 min, 4 °C) and decanted. After drying *in vacuo* for 16 h the polymers were isolated as pink powder (36 mg, 74%).

SEC (HFIP) chol-p(PMMA-MA)$_{30}$: M_n = 2,500 g/mol, M_w = 3,100 g/mol, Đ = 1.23

SEC (HFIP) chol-p(PMMA-MA)$_{30}$ (+TMR, mPEG$_{11}$-amine): M_n = 26,900 g/mol, M_w = 29,900 g/mol, Đ = 1.11

SEC (HFIP) p(PMMA-MA)$_{38}$: M_n = 3,900 g/mol, M_w = 4,700 g/mol, Đ = 1.19

SEC (HFIP) p(PMMA-MA)$_{38}$ (+TMR, mPEG$_{11}$-amine): M_n = 28,100 g/mol, M_w = 38,000 g/mol, Đ = 1.35

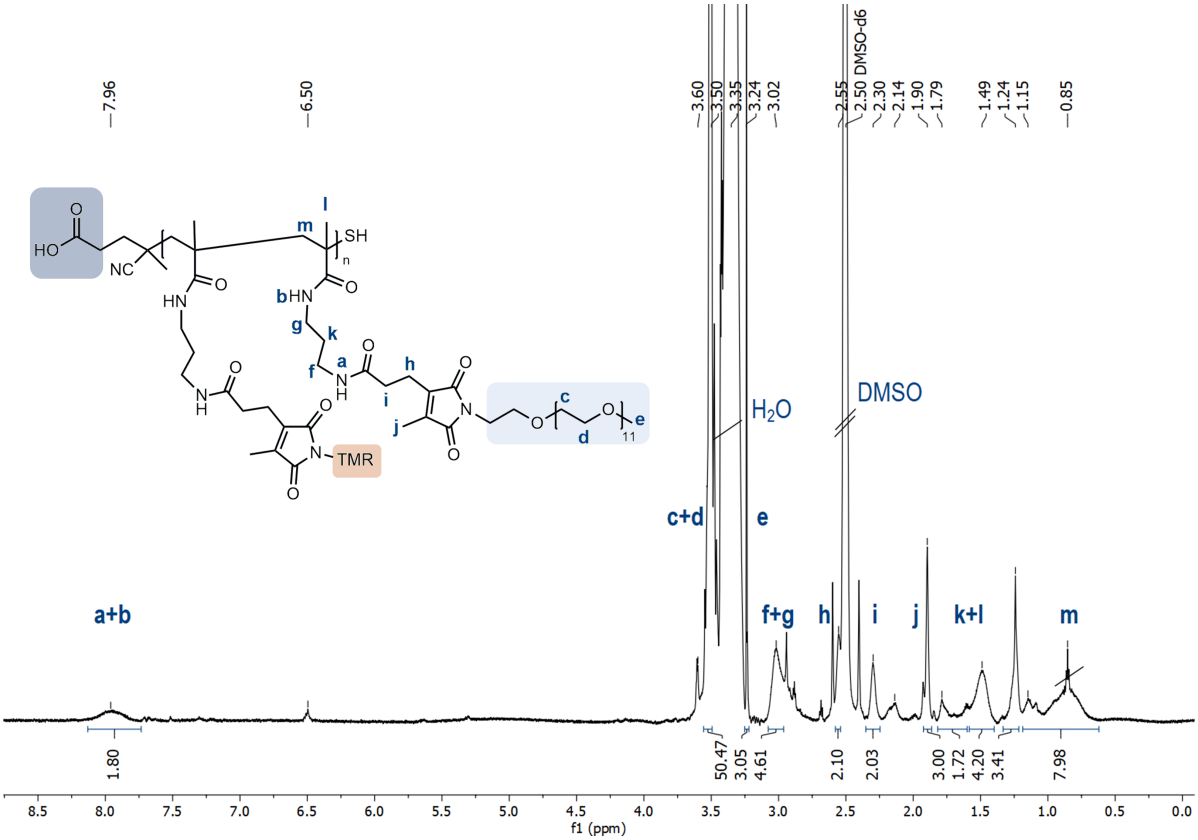

Figure S38: ^{1}H NMR spectrum (700 MHz) of the homopolymer p(PMMA-MA)$_{38}$ after modification with TMR and mPEG$_{11}$-amine in DMSO-d$_6$.

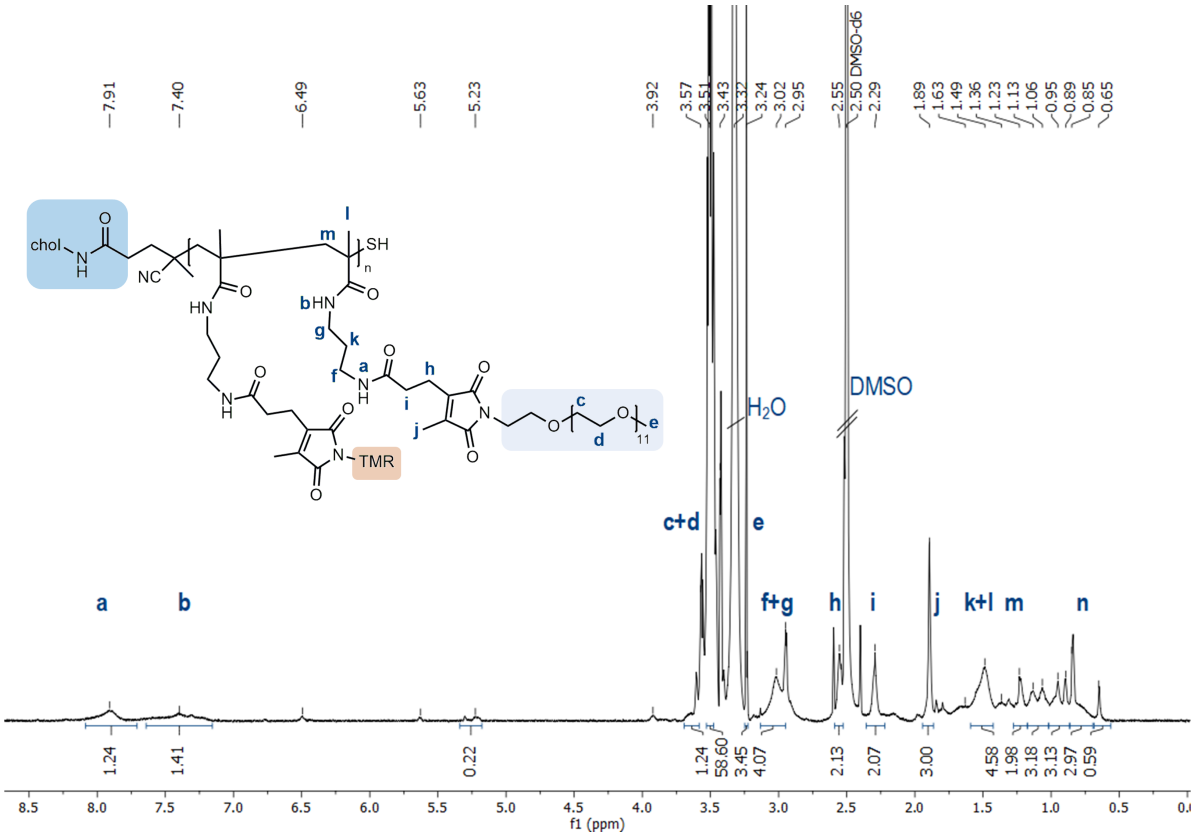

Figure S39: ^{1}H NMR spectrum (700 MHz) of the chol-popolymer chol-p(PMMA-MA)$_{30}$ after modification with TMR and mPEG$_{11}$-amine in DMSO-d$_6$.

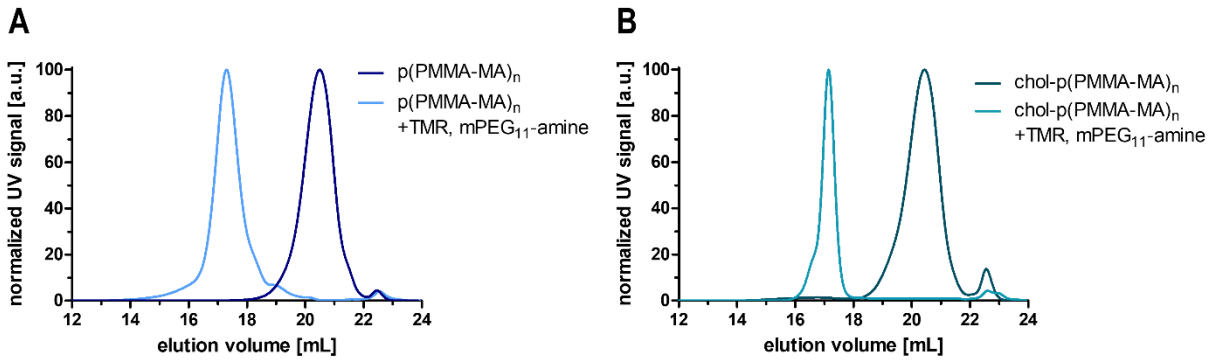

Figure S40: HFIP SEC elugrams of the homopolymer p(PMMA-MA)$_{38}$ before and after dye labeling and PEGylation (A), elugrams of the chol-polymer chol-p(PMMA-MA)$_{30}$ before and after modification (B).

3.5 Preparation of pH-Reversible and Irreversible Drug Loaded Chol-Polymers

3.5.1 Methylation of the Immune Stimulatory Drug Imidazoquinoline IMDQ

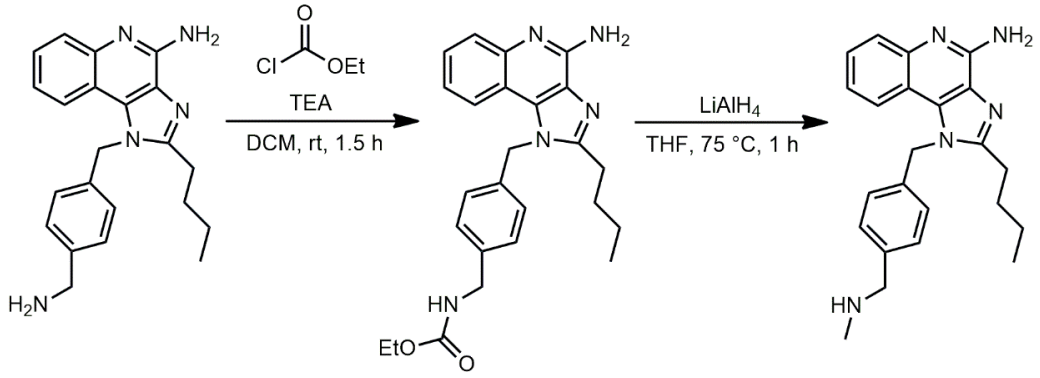

Figure S41: Synthesis of 2-butyl-1-(4-((methylamino)methyl)benzyl)-1H-imidazo[4,5-c]quinolin-4-amine (IMDQ-Me).

The synthesis of IMDQ-Me was performed similarly as previously reported[10].

^{1}H NMR (700 MHz, DMSO-d_6): δ (ppm) = 7.78 (d, J = 8.8 Hz,1H, a), 7.57 (d, J = 8.8 Hz, 1H, b), 7.37 (m, 3H, c+d), 7.01 (m, 3H, e+f), 6.53 (s, 2H, g), 5.85 (s, 2H, h), 3.73 (s, 2H, i), 2.90 (m, 2H, j), 2.30 (s, 3H, k), 1.70 (m, 2H, l), 1.38 (m, 2H, m), 1.23 (s, 1H, n), 0.86 (t, 3H, o).

ESI-MS [m/z] = 374.24 $[M+H]^+$ (calc. 374.49).

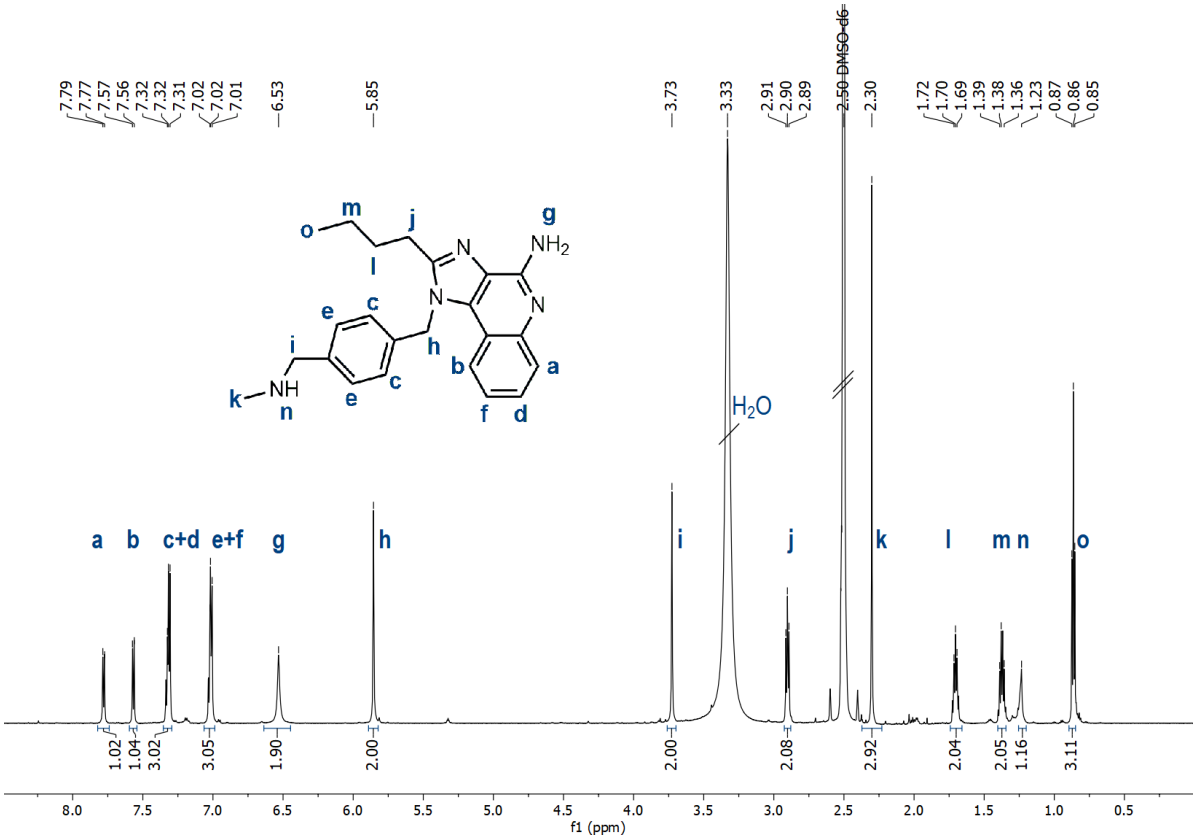

Figure S42: ^{1}H NMR spectrum (700 MHz) of 2-butyl-1-(4-((methylamino)methyl)benzyl)-1H-imidazo[4,5-c]quinolin-4-amine (IMDQ-Me) in DMSO-d_6.

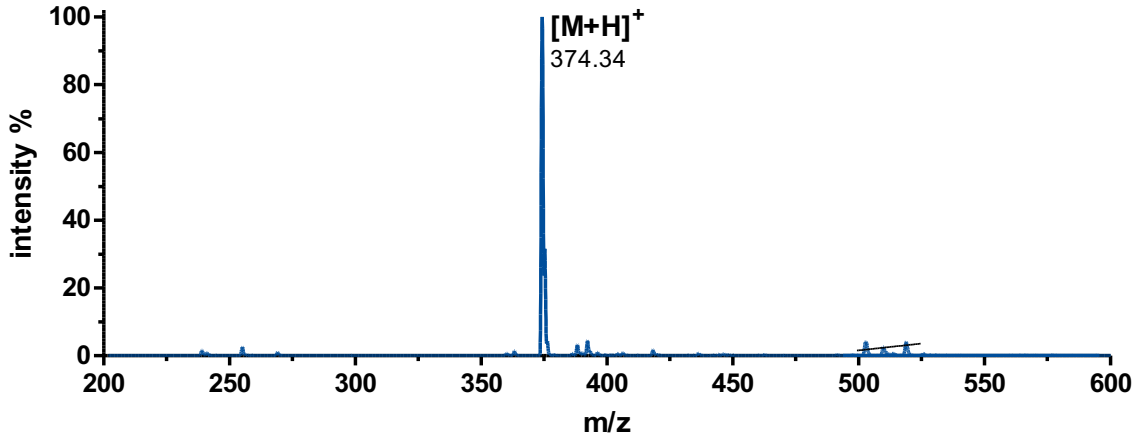

Figure S43: ESI-MS spectrum of 2-butyl-1-(4-((methylamino)methyl)benzyl)-1H-imidazo[4,5-c]quinolin-4-amine (IMDQ-Me) in MeOH (positive ion mode).

3.5.2 Polymer Drug Conjugation and PEGylation by Covalent Attachment of IMDQ or IMDQ-Me to the Chol-Polymer

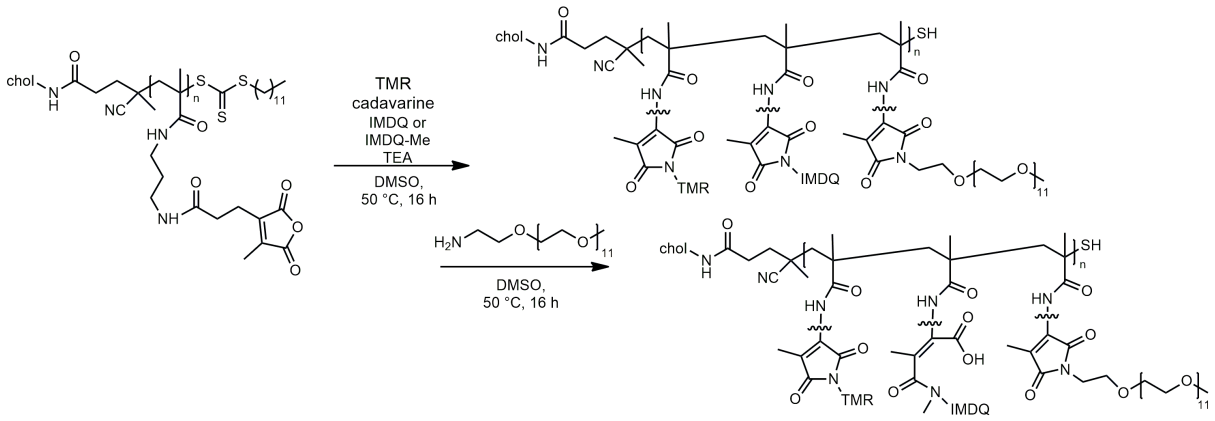

Figure S44: Schematic reaction of the chol-polymer chol-p(PMMA-MA)$_{32}$ with IMDQ or IMDQ-Me followed by PEGylation with mPEG$_{11}$-amine.

In two pre-dried Schlenk tubes the chol-polymer chol-p(PMMA-MA)$_{32}$ (2x15 mg, 1.41 µmol/45.12 µmol reactive anhydride groups, 1 eq) was dissolved in dry DMSO (0.5 mL) under nitrogen atmosphere. IMDQ (81.13 µL of a 10 mg/mL stock solution in DMSO, 2.26 µmol, 0.05 eq) or IMDQ-Me (84.30 µL of a 10 mg/mL stock solution in DMSO, 2.26 µmol, 0.05 eq) and TEA (18.76 µL, 135.4 µmol, 3 eq) were added to the solutions. The reaction mixtures were stirred at 50 °C for 16 h, followed by PEGylation with mPEG$_{11}$-amine (M_n: 0.75 kDa) (0.508 mL of a 100 mg/mL stock solution in DMSO, 67.68 µmol, 1.5 eq). The mixtures were stirred for further 16 h at 50 °C. Next, the drug-loaded polymers were precipitated three times in diethyl ether. Diethyl ether was decanted, and the obtained chol-polymers were dried under high vacuum for 16 h. The modified chol-polymers were isolated as pink powder (38 mg, 83%).

SEC (HFIP) chol-p(PMMA-MA)$_{32}$: M_n = 2,000 g/mol, M_w = 2,500 g/mol, Đ = 1.23

SEC (HFIP) chol-p(PMMA-MA)$_{32}$ (+IMDQ, TMR, mPEG$_{11}$-amine): M_n = 35,600 g/mol, M_w = 40,100 g/mol, Đ = 1.13

SEC (HFIP) p(PMMA-MA)$_{32}$ (+IMDQ-Me, TMR, mPEG$_{11}$-amine): M_n = 36,600 g/mol, M_w = 40,900 g/mol, Đ = 1.12

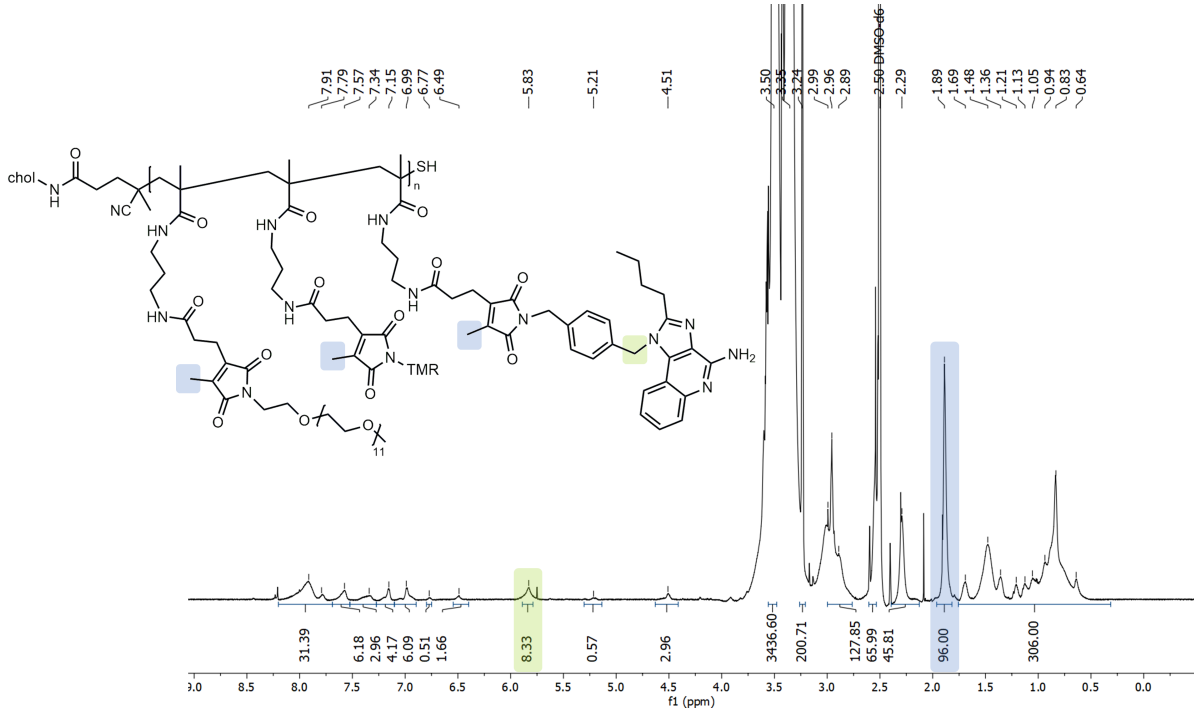

Figure S45: ^{1}H NMR spectrum (700 MHz) of the chol-p(PMMA-MA)$_{32}$ after dye labeling, drug loading with IMDQ and PEGylation with mPEG$_{11}$-amine in DMSO-d$_6$.

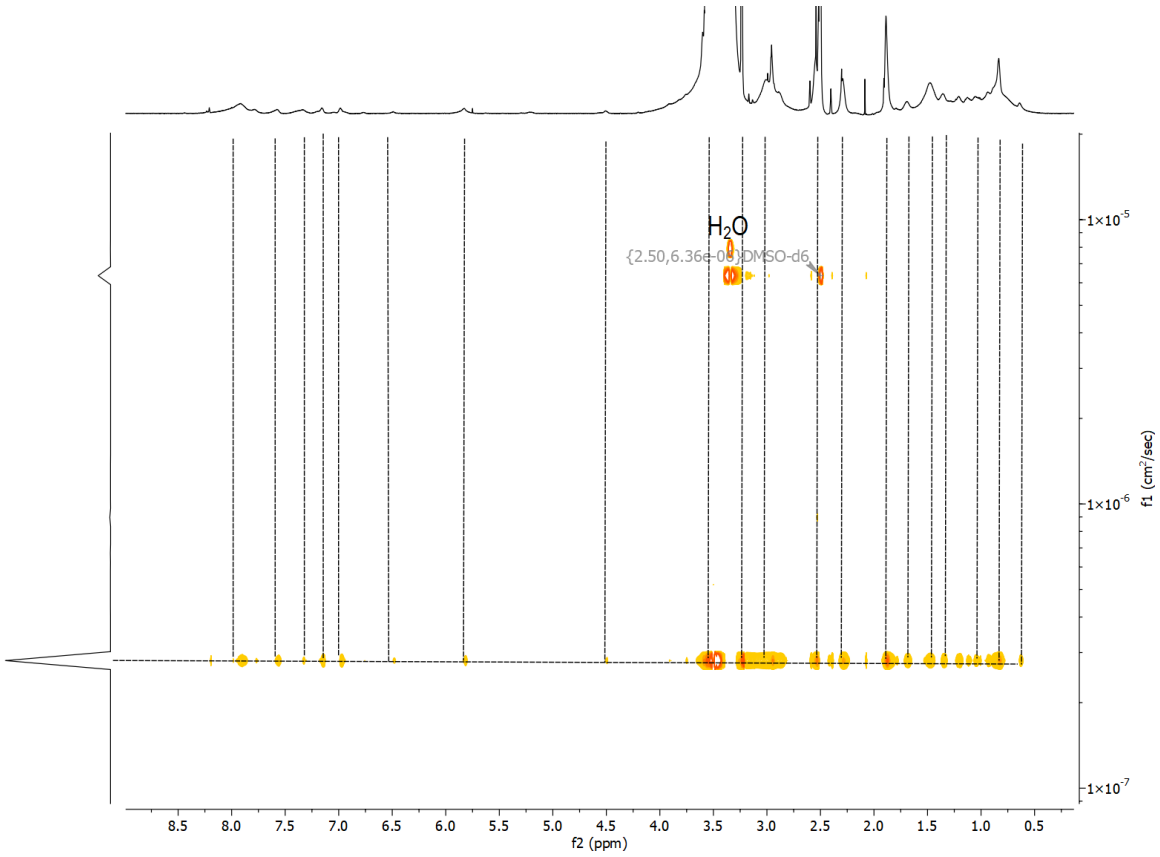

Figure S46: DOSY spectrum (700 MHz) of the chol-polymer p(PMMA-MA)$_{32}$ after dye labeling, drug loading with IMDQ and PEGylation with mPEG$_{11}$-amine in DMSO-d$_6$.

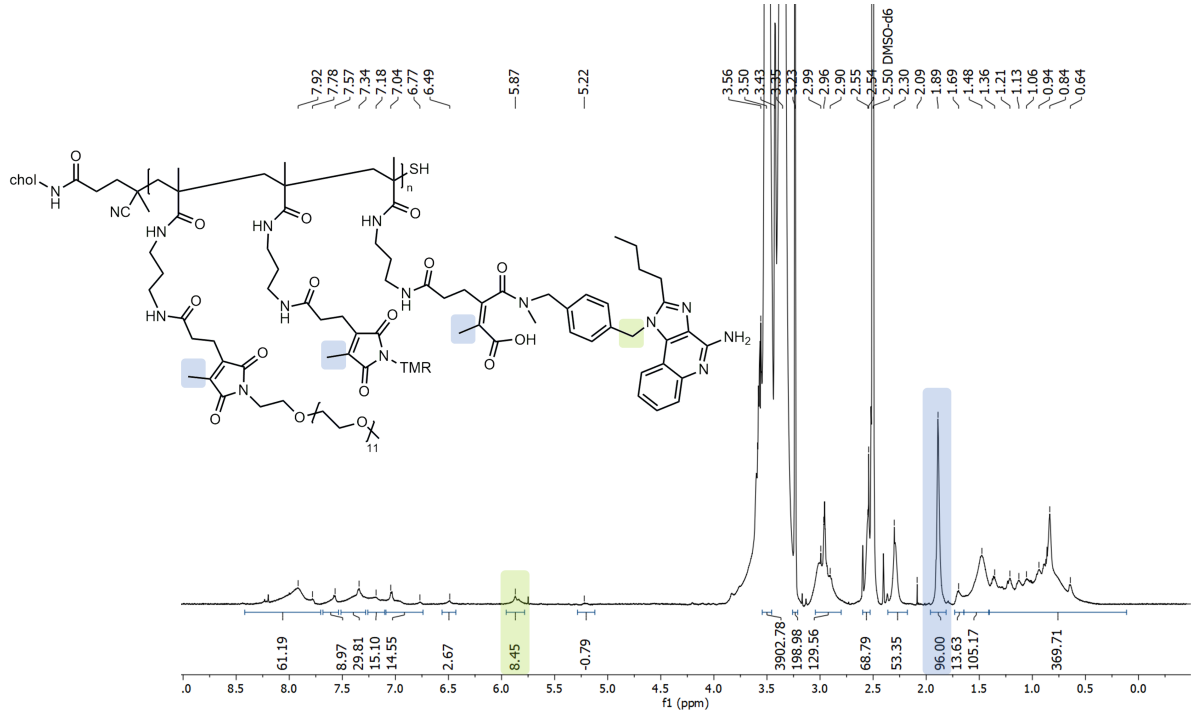

Figure S47: ^{1}H NMR spectrum (700 MHz) of the chol-polymer chol-p(PMMA-MA)$_{32}$ after dye labeling, drug loading with IMDQ-Me and PEGylation with mPEG$_{11}$-amine in DMSO-d$_6$.

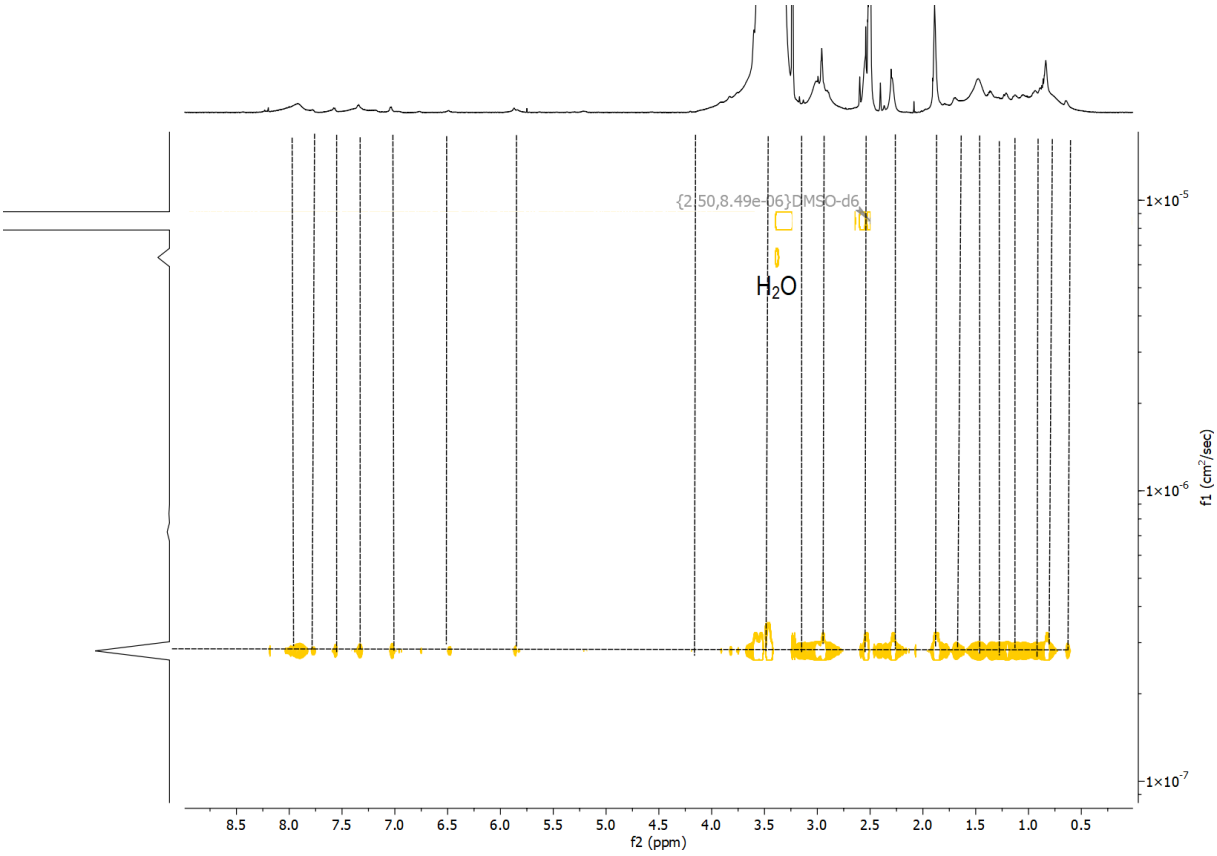

Figure S48: DOSY spectrum (700 MHz) of the chol-polymer chol-p(PMMA-MA)$_{32}$ after dye labeling, drug loading with IMDQ-Me and PEGylation with mPEG$_{11}$-amine in DMSO-d$_6$.

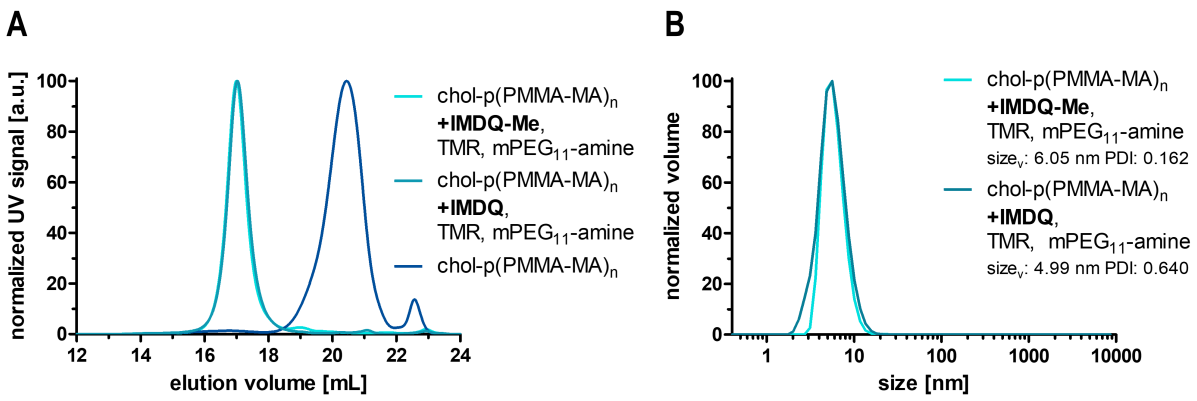

Figure S49: HFIP SEC traces of TMR-labeled, IMDQ- or IMDQ-Me-loaded and PEGylated chol-polymer (A), and the DLS volume-weighted size distribution in PBS of the fully soluble polymers do not forming micelles or aggregates (B).

3.5.3 Calculation of IMDQ or IMDQ-Me Drug Loading by ^{1}H NMR and UV-Vis Analysis

The drug load of the modified chol-polymers was determined by ^{1}H NMR and UV-Vis measurement.

For ^{1}H NMR analysis the chol-polymers were dissolved in deuterated DMSO and 3 H of =C-CH3 were used as a reference (compare Fig. S45+S47).

Calculation of the IMDQ-drug load to the chol-polymer:

Polymer DP = 32

3 H of =C-CH3 → **96 H**

IMDQ proton signal: 8.33 H → 2 H of Bn-CH2-N → 4.165 IMDQ/polymer

4.165 · 359 g/mol : 32594 g/mol → **4.6 wt%**

Calculation of the IMDQ-Me-drug load to the chol-polymer:

Polymer DP = 32

3 H of =C-CH3 → **96 H**

IMDQ-Me proton signal: 8.45 H → 2 H of Bn-CH2-N → 4.225 IMDQ-Me/polymer

4.225 · 373 g/mol : 32742 g/mol → **4.8 wt%**

For the determination of the drug load by UV-Vis analysis standard curves were created. Therefore, free drug stock solutions (10 mg/mL) were diluted in PBS and measured at different concentrations (ranging from 0.1 to 0.0031 mg/mL). According to the literature the drug load of the formulated chol-polymers was calculated.[11] The free drugs IMDQ and IMDQ-Me show a strong absorbance maximum at 322 nm, while the corresponding drug-loaded chol-polymers have absorbance maximum at 324 nm.

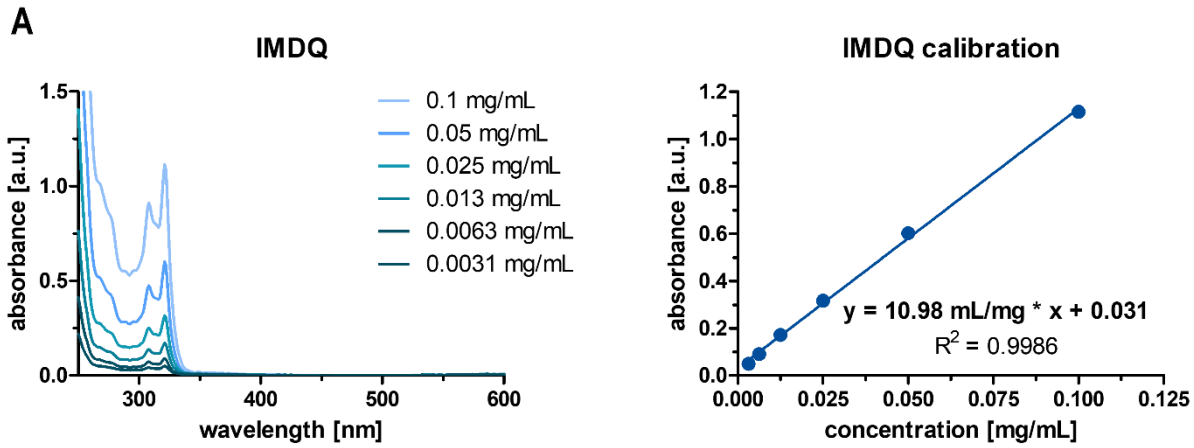

Figure S50: UV-Vis spectra of IMDQ at different concentrations in PBS (left) and the corresponding calibration curve (right).

Calculation of the IMDQ drug load to the chol-polymer:

$$X = \frac{(\Delta A - b)}{m - (A_{empty} / c)}$$

$X = 0.021 / 0.5$ mg/mL

$X =$ **4.2 wt%**

$\mathbf{\Delta A} = A_{max}$ (IMDQ chol-polymer) $- A_{max}$ (chol-polymer)
$= 0.557 - 0.307 = 0.250$

$\mathbf{b} = 0.031$

$\mathbf{m} = 10.98$ mL/mg

$\mathbf{A_{empty}} = 0.307$

$\mathbf{c} = 0.5$ mg/mL

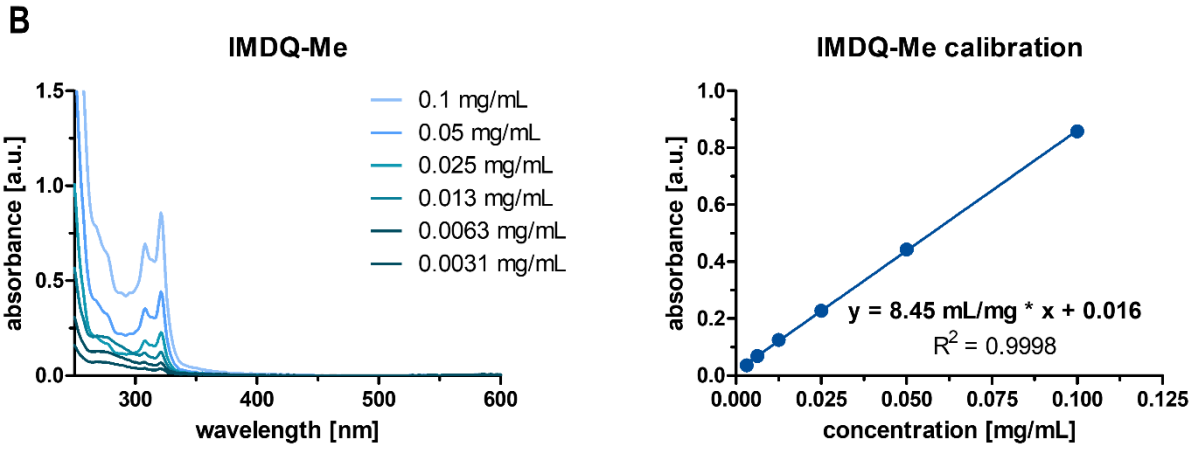

Figure S51: UV-Vis spectra of IMDQ-Me at different concentrations in PBS (left) and the corresponding calibration curve (right). Calculation of the IMDQ-Me drug load to the chol-polymer.

$$X = \frac{(\Delta A - b)}{m - (A_{empty} / c)}$$

$X = 0.023 / 0.5$ mg/mL

$X =$ **4.5 wt%**

ΔA $= A_{max}$ (IMDQ-Me chol-polymer) $- A_{max}$ (chol-polymer)
$= 0.507 - 0.307 = 0.200$

b $= 0.016$

m $= 8.45$ mL/mg

$\mathbf{A_{empty}} = 0.307$

c $= 0.5$ mg/mL

The drug load of the chol-polymers was determined by the average of ^{1}H NMR and UV-Vis analysis.

IMDQ chol-polymer drug load = **4.4 wt%**

IMDQ-Me chol-polymer drug load = **4.7 wt%**

3.5.4 PH-triggered release studies of IMDQ or IMDQ-Me from TMR-labeled and PEGylated chol-p(PMMA-MA)$_n$ polymers

The release of the reversible conjugated IMDQ-Me or irreversibly conjugated IMDQ TLR 7/8 agonist from the TMR-labeled and PEGylated chol-p(PMMA-MA)$_n$ polymer under physiologically relevant acidic conditions at pH 5.5 was analyzed by UV-Vis absorbance measurements. 2 mg of the respective polymers were dissolved in 1 mL PBS (pH 7.4) and transferred into dialyse tubes with molecular weight cut-off MWCO 1 kDa. The polymers were dialyzed against 100mM NaOAc buffer at pH 5.5 for 124 h at room temperature. At the given time points, UV-Vis spectra of the polymers were recorded by withdrawing samples from the dialysis bag (ca. 80 µL). The TMR absorbance served as internal standard while the imidazoquinoline absorbance of the TLR 7/8 agonist gradually decreased only for IMDQ-Me, while for the irreversibly conjugated IMDQ it remained constant. These results indicate a pH-triggered release only for IMDQ-Me from carrier at endolysosomal pH levels of 5.5.

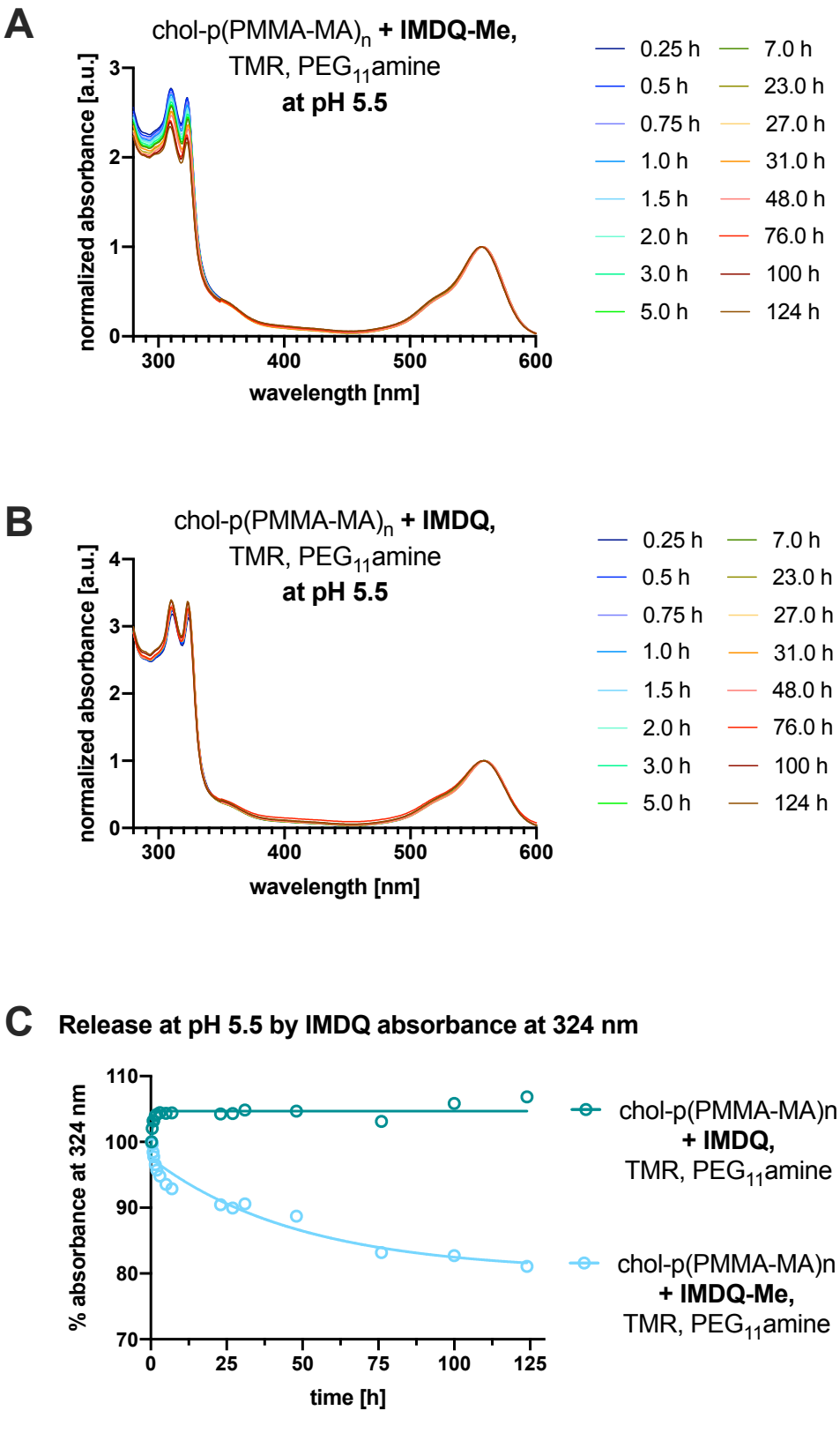

Figure S52: UV-Vis spectra of TMR-labeled, IMDQ- or IMDQ-Me-loaded and PEGylated chol-polymer during dialysis against 100 mM NaOAc buffer at pH 5.5. The reversibly IMDQ-Me-loaded chol-polymer provided a gradual decrease of the drug's absorbance (**A**) while for the irreversibly IMDQ-loaded chol-polymer the drug's absorbance remained stable (**B**). Following the drug's absorbance maxima at 324 nm, only the IMDQ-Me-loaded chol-polymer revealed a pH-responsive drug release under the applied dialysis conditions at pH 5.5.

4. *In vitro* Experiments

4.1 Determination of Chol-Polymer/Polymer Cell Uptake by Flow Cytometry (FACS)

For cellular uptake studies in RAW-Dual macrophages, cells were seeded into 24-well plates (250000 cells/well in 900 µL of culture medium) and incubated overnight. After 20 h cells were treated with 100 µL of tetramethylrhodamine-labeled, PEGylated chol-polymer or the corresponding polymer without cholesterol in PBS (yielding a concentration of 10, 30 or 100 µg/mL). The experiment was conducted for 24 h at 37 °C. Then, the medium was removed, cells were washed with 1 mL PBS and incubated with 500 µL cell dissociation buffer for 15 min at 37 °C. Afterwards, the cell suspensions were transferred into Eppendorf tubes on ice and centrifuged at 300 g for 10 minutes at 5 °C. The supernatants were removed, and the cell pellets were resuspended in 200 µL of PBS, to be analyzed on a BD Accuri C6 (BD Biosciences). All data were processed by FlowJo Software and all samples were run in triplicates (n=3).

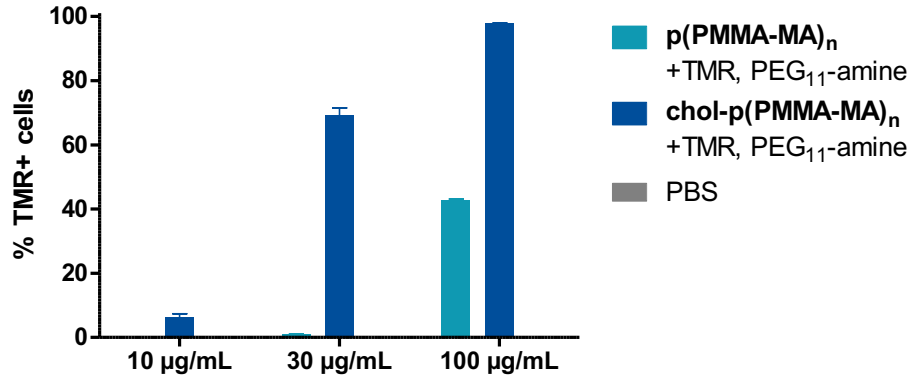

Figure S53: Flow cytometric uptake analysis in RAW-Dual macrophages incubated with PBS (control), TMR-labeled polymer PEGylated with PEG_{11}-amine or TMR-labeled chol-polymer PEGylated with PEG_{11}-amine at 10, 30 and 100 µg/mL for 24 h. Flow cytometry measurements yielding the percentage of TMR positive cells (% TMR+ cells).

Fluorescent Confocal Microscopy Imaging

For fluorescent confocal laser scanning microscopy RAW-Dual macrophages were seeded in an Ibidi µ-slide 8-well chamber (50000 cells/well in 180 µL culture medium) and left to adhere overnight. Next, the cells were incubated with 20 µL TMR-labeled, PEGylated chol-polymer or the corresponding polymer solutions in PBS (total concentration of 100 µg/mL) for 24 h at 37 °C. Then, the medium was removed, and the cells were three times washed with PBS and fixed with 200 µL 4% paraformaldehyde solution for 15 min at 37 °C. Afterwards, the cells were washed again three times with PBS and cell nuclei were stained with 125 µL 4′,6-diamidino-2-

phenylindole (DAPI) (80 µg/mL in PBS) for 20 min at room temperature. Finally, after washing with PBS three times the samples were stored under aqueous mounting medium and analyzed on a STELLARIS 8 Leica DMi8 confocal microscope (Wetzlar, Germany) with a HC PL APO CS2 40x/1.25 GLYC oil immersion objective. All images were processes by Leica Application Suite X 3.7.4.23463 of the Leica Microsystem.

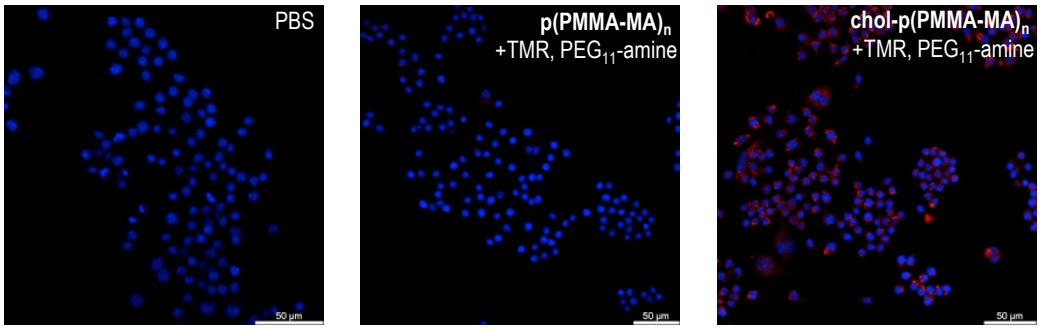

Figure S54: Confocal microscopy images of RAW-Dual macrophages incubated with PBS (control), TMR-labeled, PEGylated **polymer** or TMR-labeled, PEGylated **chol-polymer** at 100 µg/mL for 24 h (blue: nuclei stained with Hoechst 33258, red: TMR-labeled polymer).

4.3 Determination of the Cellular Metabolic Activity by MTT Assay on Stimulated RAW-Dual Macrophages

Determination of the cellular metabolic activity can be used as an indicator for cell viability and cytotoxicity. Therefore, RAW-Dual macrophages were seeded in a 96-well plate at a concentration of 90000 cells/well in 180 µL and left to adhere for 20 h. The cells were treated with 20 µL IMDQ- or IMDQ-Me-loaded chol-polymer, free drugs, or chol-polymer solution at given concentration. All experiments were performed in quadruplicates. After 24 h of incubation time at 37 °C 30 µL of a 2 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide solution in PBS was added, and cells were incubated for further 1.5 h at 37 °C. Formed formazan crystals were dissolved by addition of 100 µL of 10% m/v SDS/0.01 M HCl solution and incubated overnight at 37 °C. Quantification of the metabolic activity was done by measuring the absorbance at 570 nm using a plate reader.

4.4 RAW-Dual Macrophage TLR Reporter Assay (QUANTI-Blue Assay)

To analyze the immune modulatory properties of IMDQ/IMDQ-Me-conjugated chol-polymers a TLR reporter assay was performed as recommended by the manufacturer (InvivoGen). The IMDQ-induced downstream activation of NF-κB/AP-1 was determined by the secretion of embryonic alkaline phosphatase (SEAP). RAW-Dual cells were seeded similarly as previously reported for the MTT assay and incubated with 20 µL of the respective solutions at the given

IMDQ/IMDQ-Me concentration. After 24 h incubation at 37 °C, 50 µL of the supernatant was collected and the SEAP levels were probed by QUANTI-Blue Assay. After incubation for 2 h at 37 °C with 150 µL QUANTI-Blue solution, wells were read-out for their absorbance at 620 nm using a plate reader. All experiments were conducted at n=4.

4.5 BMDCs (Bone marrow derived dendritic cells)

Bone marrow (BM) cells were isolated from femurs and tibiae of C57BL/6 mice followed by resuspension in IMDM medium containing 5% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µM β-Mercaptoethanol (all reagents were obtained from Sigma-Aldrich, Deisenhofen, Germany), supplemented with 10 ng/ml recombinant murine GM-CSF (R&D Systems, Wiesbaden, Germany). Next, BM cells were seeded in 12-well suspension cell culture cluster plates ($2x10^5$/ml). After 3 and 6 days the culture media was replenished. On days 7-8, the differentiated cells were treated with IMDQ- or IMDQ-Me-loaded chol-polymer, soluble IMDQ and IMDQ-Me, or chol-polymer solution. After incubation for 24 h, samples were harvested and analyzed by flow cytometry. All experiments were performed in triplicates.

4.5.1 Flow Cytometry

Prior to flow cytometric analysis all samples were pretreated with a rat anti-mouse CD16/CD32 antibody (15 min, 4 °C) to prevent Fc-mediated binding of subsequently applied antibody (clone 2.4G2; 15 min, room temperature). Afterwards, samples were incubated (20 min, 4 °C) with distinct sets of fluorescence-labeled antibodies as indicated in Table S1. Samples were left untreated, incubated with the according cholesterol polymer samples alone or with single antibody serving as controls. Subsequently, FVD was added to all samples to delineate dead cells. Antibodies were purchased from BD Biosciences, Thermo Fisher or BioLegend. FVD was obtained from Thermo Fisher. Afterwards, samples were fixed (4% paraformaldehyde, 2mM EDTA) and analyzed in an Attune NxT flow cytometer (Thermo Fisher). The resulting data were processed by Attune Nxt Software v3.1.1. following the gating strategy (Figure 50).

Table S1: Antibodies used to characterize BMDCs and their maturation/activation.

Cell Type	Cell Marker	Antibody	Fluorochrome	Clone
BMDC GM-CSF	DC	CD11c	PE-Cy7, BV421	N418
Others Cell Staining		**Antibody**	**Fluorochrome**	**Clone**
Activation		CD80	PE, PerCP-eFl780	16-10A1
		CD86	FITC	GL1
Fc Block		CD16/32	n.a.	2.4G2
Live/Dead		Fixable Viability Dye	eFl450, eFl780	n.a.

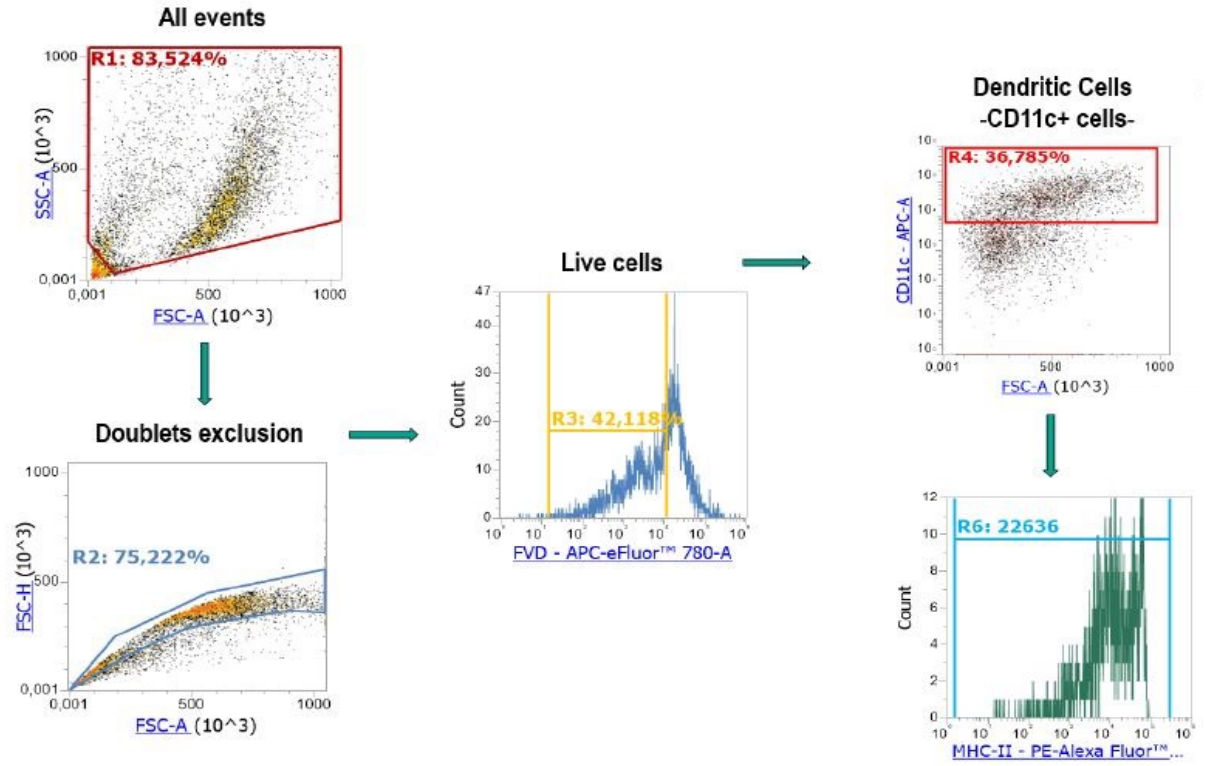

Figure S55: Gating strategy of BMDCs-GM CSF. After exclusion of debris, doublets, and dead cells (fixable viability dye $[FVD]^-$), $CD11c^+$ cells were characterized for the activation markers CD80 and CD86.

4.5.2 Cytometric Bead Assay

Supernatants of cell cultures were retrieved and stored at −20 °C for subsequent analysis. TNFα, IL-1β and IL-6 cytokines were quantified using a Cytometric Bead Assay (CBA; BD Biosciences) as recommended by the manufacturer. For this purpose, the kit used a bead-based multiplex assay with fluorescence−encoded beads that were conjugated with cytokine-specific capture antibodies. Samples were mixed with capture beads, subsequently incubated with detection antibodies and then with PE-conjugated detection antibodies (all at room temperature protected from light under shaking) and subjected to flow cytometric analysis. The obtained results were analyzed using FCAP Array Analysis Software v.1.0.1 (BD Biosciences).

5. *In Vivo* Experiments

5.1 Bioactivity of IMDQ- or IMDQ-Me-Conjugated Chol-Polymers

To study the immune stimulatory effect of IMDQ- or IMDQ-Me-conjugated chol-polymers, heterozygous BALB/c IFN-β (IFN-$\beta^{+/\Delta\beta\text{- luc}}$) reporter mice in the range of 7–9 weeks were housed in individual ventilated cages and given ad libitum access to food and water. 20 µL of the IMDQ- or IMDQ-Me-conjugated chol-polymers as well as soluble IMDQ and IMDQ-Me were injected subcutaneously in the footpad at an equivalent dose of 10 µg IMDQ or IMDQ-Me. Additional, mice treated with PBS or polymer with or without cholesterol served as controls (n = 3 or 4). For subsequent *in vivo* bioactivity imaging, mice were injected subcutaneously with 200 µL D-luciferin (15 mg/ mL, Gold Biotechnology, USA) at 4 and 24 h, and *in vivo* luminescence imaging was recorded 12 min later using the IVIS Lumina II imaging system from PerkinElmer, (Waltham, MA, USA). Photon flux was analyzed by Living Image 4.4 software from Caliper life sciences (Hopkinton, MA, USA).

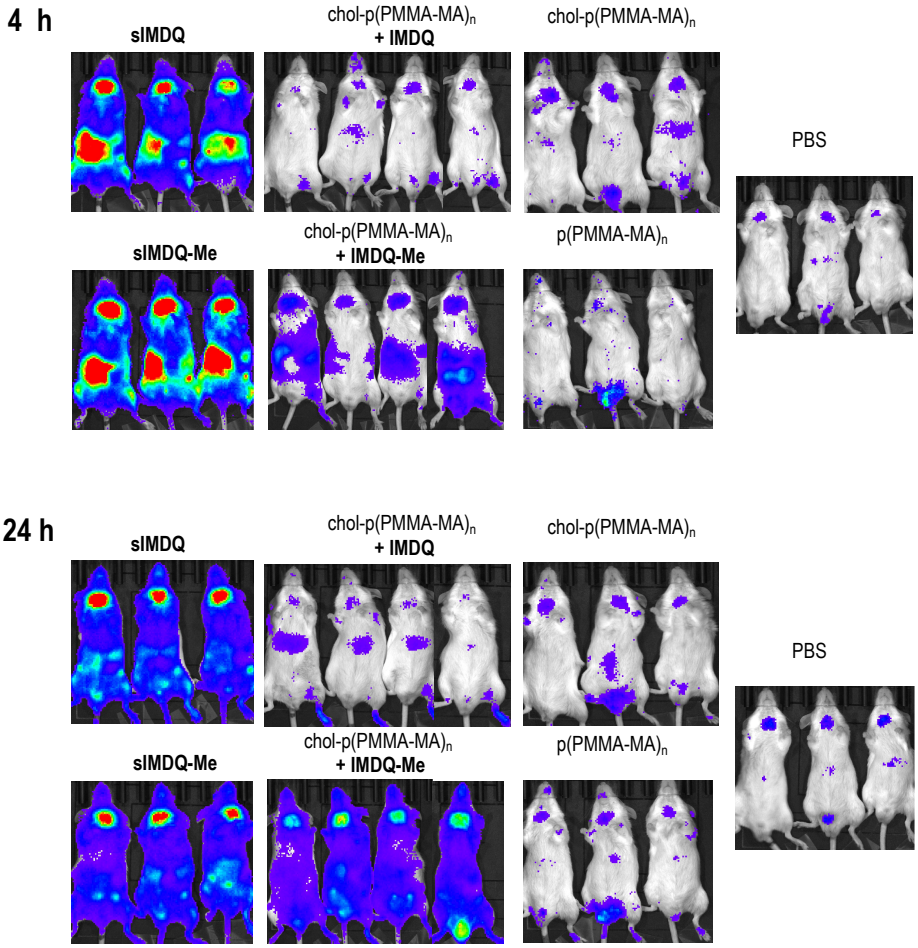

Figure S56: In vivo bioactivity of soluble and chol-p(PMMA-MA)$_n$ conjugated IMDQ and IMDQ-Me as well as chol-p(PMMA-MA)$_n$ and p(PMMA-MA)$_n$ after footpad injection into heterozygous BALB/c IFN-β (IFN-$\beta^{+/\Delta\beta\text{-luc}}$) reporter mice (n = 3 or 4).

5.2 Analysis of Lymphocyte Targeting and Activation

Heterozygous BALB/c IFN-β (IFN-β+/Δβ-luc) reporter mice were subcutaneously injected in the footpad with 10 µg IMDQ or IMDQ-Me (soluble or bound to the chol-polymers in 20 µL of PBS, n=3) and sacrificed after 48 h. Popliteal lymph nodes were isolated for flow cytometry. A single cell suspension was prepared form three of the dissected lymph nodes for analysis by flow cytometry. The isolated lymph nodes were collected in ice cold PBS, smashed through 70 µm cell strainers, washed with PBS and stained for 30 min at 4 °C with a live-dead stain and the following primary labeled monoclonal antibodies: CD3, CD4, CD8, CD11c, CD11b, CD19, CD45, CD69, CD86, CD172a, CCR7, XCR7, F4-80, and MHC-II. 123count beads were added to determine cellularity prior to acquiring them by flow cytometry (BD FACS Aria). Analyses were done using the FlowJo software package according to the gating procedure.

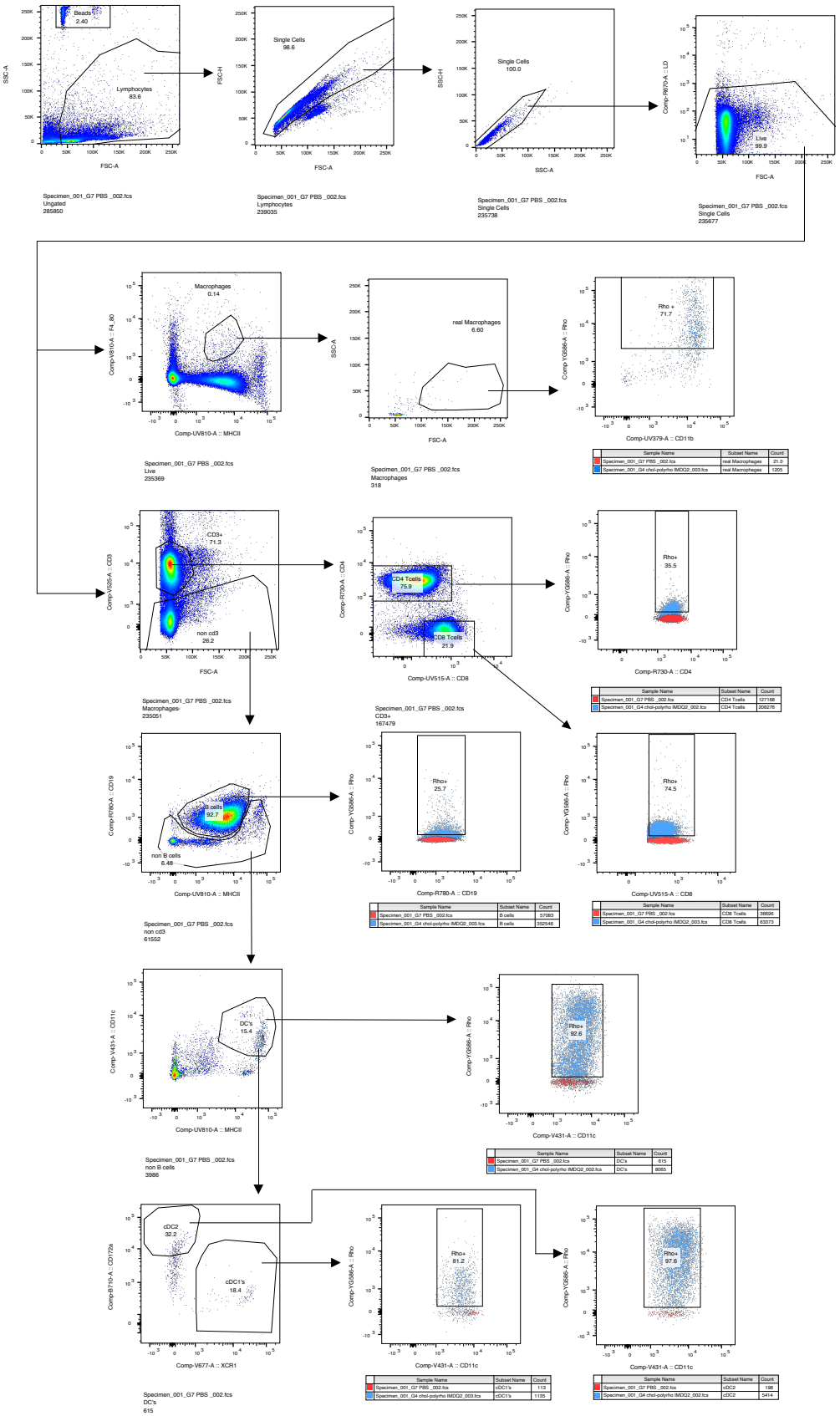

Figure S57: Gating procedure for flow cytometry analysis of popliteal lymph node immune cell targeting (and maturation).

6. Statistical Analyses

All data are shown as mean values ± standard deviation (mean ± SD). Samples sizes (n) were mentioned for each data set. To calculate statistical significance of the mean values, unpaired Student's t-test with Welch's correction was performed using Graph Pad Prism 8 software for deriving the respective p-values.

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