Electronic Supplementary Information (ESI)

pH-responsive poly(4-hydroxybenzoyl methacrylates) – design and engineering of intelligent drug delivery nanovectors

Francesca Mastrotto[a,b], Yi Lin Lee[b], Stefano Salmaso[b], Cameron Alexander[a], Paolo Caliceti[b,*] and Giuseppe Mantovani[a,*]

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1. Materials

Methacryloyl chloride (97%), 3-chloro-4-hydroxybenzoic acid hemihydrate (98%), 3,5-dichloro-4-hydroxybenzoic acid (97%) 3-chloro-4-methoxybenzoic acid (≥98%) were purchased from Alfa Aesar. Potassium hexacyanoferrate (III) (>99%), 2-hydroxyethyl methacrylate (HEMA, 97%), poly(ethylene glycol) methyl ether methacrylate ($mPEGMA_{475}$), mercaptoethanol (≥99%), *N,N′*-dicyclohexylcarbodiimide (DCC, 99%), 4-(dimethylamino)pyridine (DMAP, ≥99%), 4, 4'-azobis (4-cyanovaleric acid) (≥98%), pyrene (≥97%) and 6-(*p*-toluidino)-2-naphthalenesulfonic acid sodium salt (TNS) were purchased from Sigma-Aldrich and used for synthesis without further purification except from 2,2′-azobis(2-methylpropionitrile) (AIBN, Sigma-Aldrich, 98%) which was recrystallized from methanol. Silica gel was purchased by Acros Organics. Acetonitrile (ACN, 99.8%), diethyl ether (anhydrous, ≥99%) dichloromethane (DCM, 99.8%), *N,N*-dimethylformamide (DMF, anhydrous, 99.8%), dimethylsulfoxide (DMSO) (99%), ethyl acetate (EtOAc, anhydrous, 99.8%), methanol (MeOH, ≥99.9%), petroleum ether, tetrahydrofuran (THF, anhydrous, ≥99.9%), were purchased from Sigma-Aldrich and used as received. Anhydrous solvents were used as received and stored under dry and inert atmosphere. Doxorubicin hydrochloride salt (>99%) was obtained by LC Laboratories.

2. Instrumentation

2.1 Analysis. The measurements were carried out using NaCl discs and NICOLET IR200 FT-IR spectrometer (Thermo Fisher Scientific). The mass spectrometric analyses were carried out using a Mariner ESI-TOF spectrometer (Thermo Fisher Scientific). The ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded at room temperature on a 400 MHz (Bruker DPX400 Ultrashield) using deuterated solvents ($CDCl_3$ or DMSO-d_6). All chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as an internal reference.

2.2 Gel Permeation Chromatography. The polymer molecular weights were determined by gel permeation chromatography (GPC) using a Polymer Laboratories GPC 50 system (Polymer Laboratories) equipped with two columns connected in series (Agilent PLgel 5 μm Mixed D, 7,5 x 300 mm) and an RI detector, eluting with DMF + 0.1 % w/w LiBr at flow rate of 1 mL min^{-1}. The molecular weights and polidispersity indices of the polymers were calculated according to a calibration curve obtained with PMMA narrow standards (162-371,000 g mol^{-1}). Data was elaborated with Cirrus GPC/SEC 3.0 software.

3. Methods

3.1 $PEGMA_{11}$-*b*-MCH_{38} P15 polymersome membrane permeability study. A permeability test was carried out using TNS, a fluorescent probe that is only weakly fluorescent in aqueous buffer while it becomes highly fluorescent in hydrophobic environment such as the hydrophobic bilayer of polymersomes.[23] TNS did not show significant incorporation within the micellar core of $PEGMA_{11}$-*b*-MCH_{21} **P14** and $PEGMA_{11}$-*b*-MCM_{20} **P16**.

Table S1 Size of poly(PEGMA-*b*-MCH) and poly(PEGMA-*b*-MCM) assemblies (1.0 mg mL^{-1}) in PBS at pH 7.4

Code	Polymers	Size (nm)	PDI	Aggregates[a]
P14	$PEGMA_{11}$-*b*-MCH_{21}	41.9±0.6	0.128	Micelles
P15	$PEGMA_{11}$-*b*-MCH_{38}	201.1±3.5	0.100	Vescicles
P16	$PEGMA_{11}$-*b*-MCM_{20}	24.2±2.4	0.157	Micelles

[a]as assessed by TEM analysis.

To a colloidal suspension of **P15** polymersomes (10 mL of 0.3 mg mL^{-1}) prepared as described in the *Drug loading experiments* paragraph in the main article 2(p-toluidino)naphthalene-6-sulfonic acid (TNS, 1 mg) was added. The pH was adjusted to 8.0 with 1 N NaOH, the samples were titrated with 0.5 N HCl. Fluorescence spectra pH were recorded in the of 350-500 nm λ_{em} range. The intensity values at 445 nm were plotted against the pH (Figure 4 of the main article).

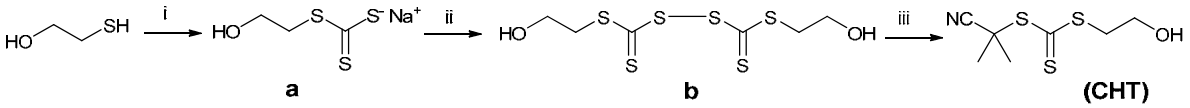
3.2 Quantification of tamoxifen and doxorubicin HCl loading in poly(PEGMA-*b*-MCH) and poly(PEGMA-*b*-MCM) nanovectors. Spectrophotometric quantification of doxorubicin HCl solutions was performed using a molar extinction coefficient of 11500 M^{-1} cm^{-1} at λ=480 nm. The amount of doxorubicin HCl loaded in polymer assemblies was quantified via fluorimetric analysis of micelle/polymersome samples diluted in DMSO to disassemble the nanovectors and then further diluted in 0.02 M phosphate buffer, 0.15 M NaCl pH 7.4. The amount of loaded drug was determined using a calibration curve obtained with standard solutions of doxorubicin HCl in 0.02 M phosphate buffer, 0.15 M NaCl pH 7.4 [y = 3.97 x (doxorubicin HCl ng mL^{-1}) + 38.219 R^2 = 0.9929, detection lower limit 5 ng mL^{-1}] as a reference.

Tamoxifen loading capacity was assessed by RP-HPLC. A reversed-phase C_{18} column (Luna, 5 μm, 250 x 46 mm, Phenomenex) was used as stationary phase eluted with milliQ water + 0.05% TFA (eluent A), acetonitrile + 0.05% TFA (eluent B) as mobile phases at the flow rate of 1 mL min^{-1} with a gradient going from 40% to 90% of eluent B in 10 minutes. The system was equipped with an UV detector (Jasco UV 2077 Plus) set at λ=275 nm. In a typical experiment 100 μL of Tamoxifen-loaded micelles in 20 mM Na_2HPO_4, 150 mM NaCl pH 7.4 were centrifuged at 10000 rpm for 5 minutes

and filtered through Corning Costar® Spin-X® centrifuge tube equipped with cellulose acetate membrane filters with pore size of 0.45 µm. Polymersomes were purified through a SEC-column packed with a sephadex G25 superfine resin using PBS pH 7.4 as the mobile phase. 50 µL of purified suspensions were diluted with 950 µL of MeOH to disassemble the polymer nanovectors, and the resulting mixtures centrifuged at 10000 rpm for 5 minutes twice. Finally, 20 µL of supernatant were analyzed by RP-HPLC. The amount of loaded drug was determined using a calibration curve obtained with standard solutions of Tamoxifen [$y = 41020 \times (\text{Tamoxifen } \mu g\ mL^{-1}) + 315.44$ $R^2 = 0.9996$, detection limit: 10 ng mL^{-1}].

4. Synthesis

4.1 Synthesis of 2-cyanopropan-2-yl 2-hydroxyethyl carbonotrithioate (CHT) chain transfer agent. The synthesis of the RAFT chain transfer agent, 2-cyanopropan-2-yl 2-hydroxyethyl carbonotrithioate (CHT) (scheme S1) was performed by an adaptation of a standard according to modified methods described in the literature.[1, 2]



Scheme S1: Synthesis of 2-cyanopropan-2-yl 2-hydroxyethyl carbonotrithioate (CHT) chain transfer agent. *Reagents and conditions:* (i) 1. NaH; 2. CS_2, 0°C; (ii) $K_3Fe(CN)_6$, H_2O; (iii) AIBN, EtOAc, reflux, 17 h.

Sodium 2-hydroxyethyl carbonotrithioate (a). NaH (60 wt % in mineral oil, 2.82 g, 70.4 mmol) was dispersed in 50 mL of diethyl ether and cooled to 5°C in an ice bath. Mercaptoethanol (4.49 mL, 0.064 mol) was added dropwise under stirring to the organic suspension and the mixture was stirred for 10 minutes, then CS_2 (5.8 mL, 96 mmol) was added dropwise to the suspension and the

reaction was stirred at ambient temperature for one hour. The resulting yellow precipitate was obtained and recovered by filtration, washed with diethyl ether, and finally desiccated under reduced pressure, to give the intermediate (a) which was used for the next step without further purification. Yield: 7.53 g, 43 mmol, 67%.

^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 3.48 (t, J = 7.2 Hz, 2H, CH_2-S), 3 .66 (td, J = 7.2, 5.5 Hz, 2H, CH_2-O), 5.14 (t, J = 5.5 Hz, 1H, OH).

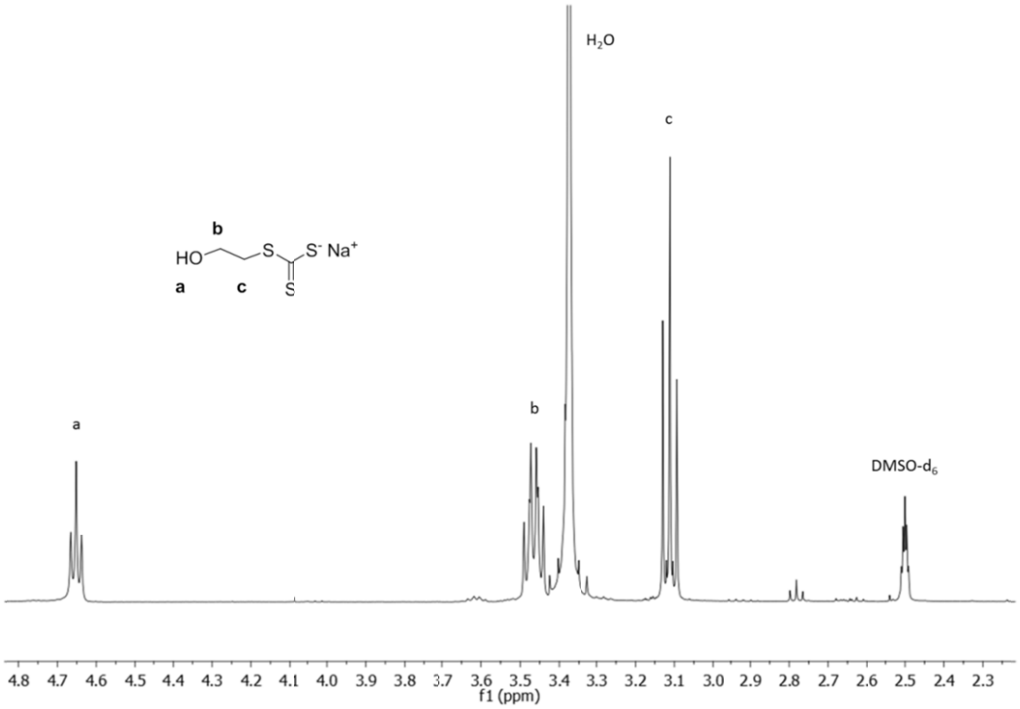


Figure S1. ^{1}H NMR spectrum of unpurified intermediate *(a)* in DMSO-d_6.

Dithiobis-2-hydroxyethyl carbonotrithioate disulfide (b). Sodium 2-hydroxyethyl carbonotrithioate intermediate (*a*) (7.5 g, 43 mmol) was dissolved in 100 mL of water and of $K_3Fe(CN)_6$ (16 g, 47 mmol) was slowly added. The resulting viscous reddish orange precipitate was extracted from the aqueous layer with diethyl ether (50 mL). The extraction process was repeated four times and the organic layers, combined, were dried over $MgSO_4$, The mixture was filtered and the solvent evaporated under reduced pressure to give (b) (5.8 g, 19 mmol, 89 %) as an orange viscous oil which was used for the next step without further purification.

^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 3.48 (t, J = 6.1 Hz, 4H, CH_2-S), 3 .66 (t, J = 6.1 Hz, 4H, CH_2-O), 5.14 (broad s, 2H, OH).

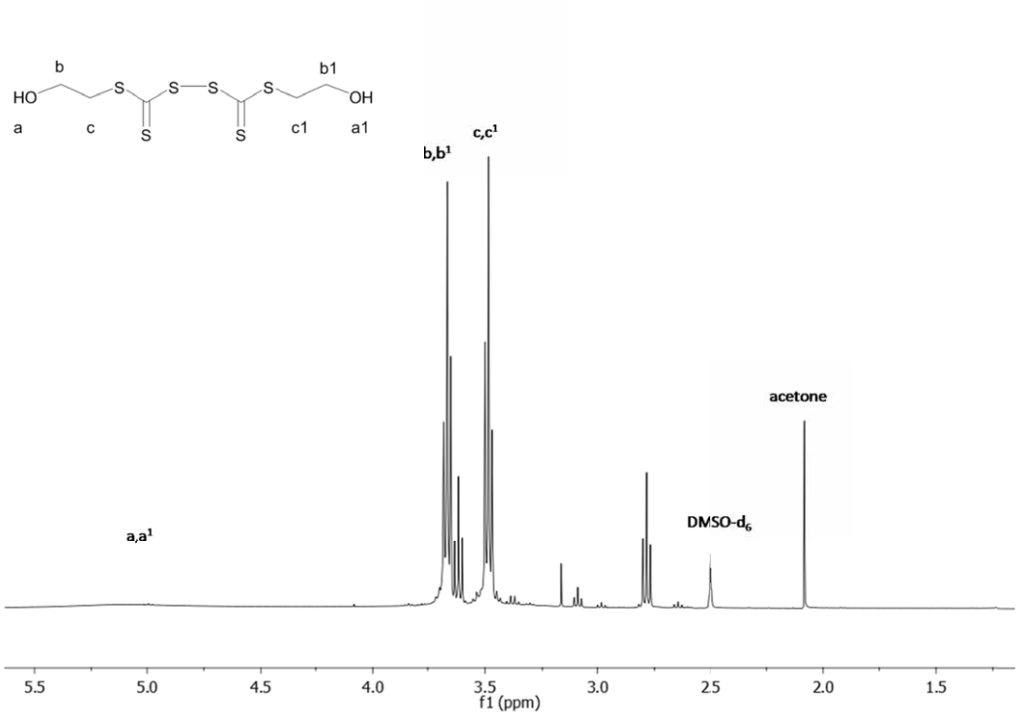


Figure S2. ^{1}H NMR spectrum of unpurified intermediate *(b)* in DMSO-d_6.

2-cyanopropan-2-yl 2-hydroxyethyl carbonotrithioate (CHT). Dithiobis-2-hydroxyethyl carbonotrithioate disulfide intermediate (*b)*, (5.8 g, 19 mmol) and AIBN (4.7 g, 28 mmol) were dissolved in EtOAc (60 mL) and the resulting solution was degassed for 30 minutes by bubbling argon under stirring. The reaction mixture was placed in an oil bath and refluxed at 80°C for 17 hours. The reaction was monitored by TLC on silica gel (petroleum ether/EtOAc, 7:3 v/v) and ^{1}H NMR in DMSO-d_6. The organic solvent was removed under reduced pressure and the yellow oily residue was purified by flash chromatography (silicagel 60, 35-70 μm) using petroleum ether/EtOAc 7:3 (vol/vol) as the eluent. After removal of the solvent from the relevant fractions the volatiles were evaporated under reduced pressure to give analytically pure CHT (6.6 g, 30 mmol, 79 %) as an orange oil.

ESI-TOF mass spectrometry: expected m/z $[M-H]^+$ theor. 222.01, found 222.01 u.m.a.. FT-IR ν 3429, 2929, 2234, 1662, 1461, 1387, 1285, 1203, 1132, 1080, 945, 874, 806 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.88 (s, 6H, CH_3), 3 .58 (t, J = 6.0 Hz, 2H, CH_2-S), 3.89 (t, J = 6.0 Hz, 2H, O-CH_2), 2 (bs, 1H, OH). $^{13}C\{^1H\}$ NMR (400 MHz, $CDCl_3$, δ, ppm): 26.87 (2C, CH_3), 38.86 (1C, C-$(CH_3)_2$), 42.51 (1C, CH_2-S), 60.04 (1C, CH_2-OH), 120.13 (1C, C≡N), 217.53 (1C, C=S).

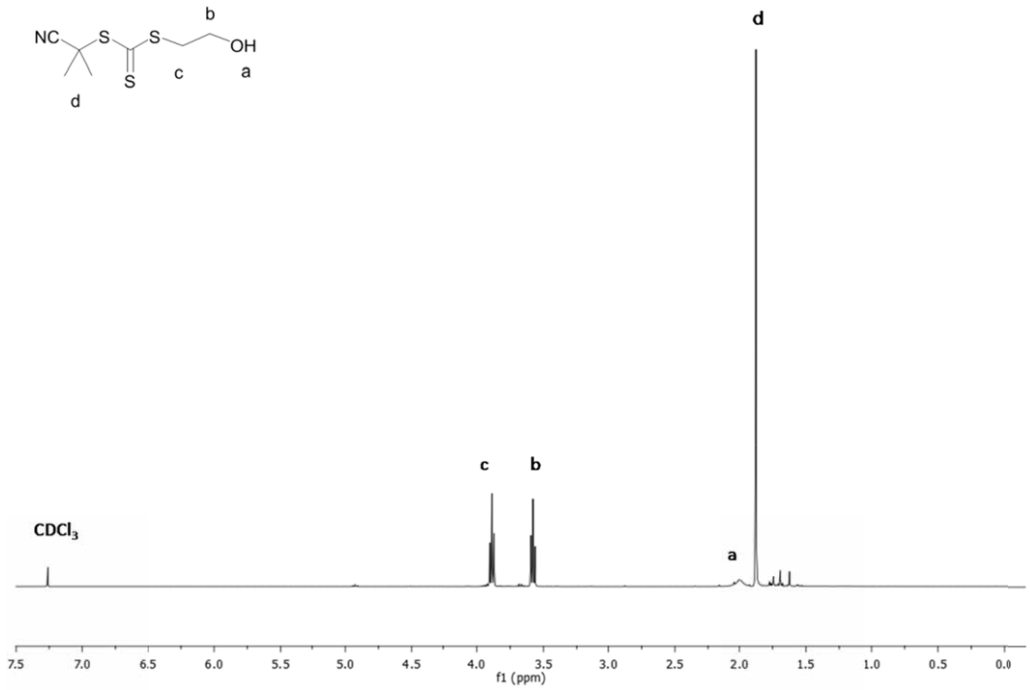


Figure S3. ^1H NMR spectrum of purified 2-cyanopropan-2-yl 2-hydroxyethyl carbonotrithioate (CHT) in $CDCl_3$.

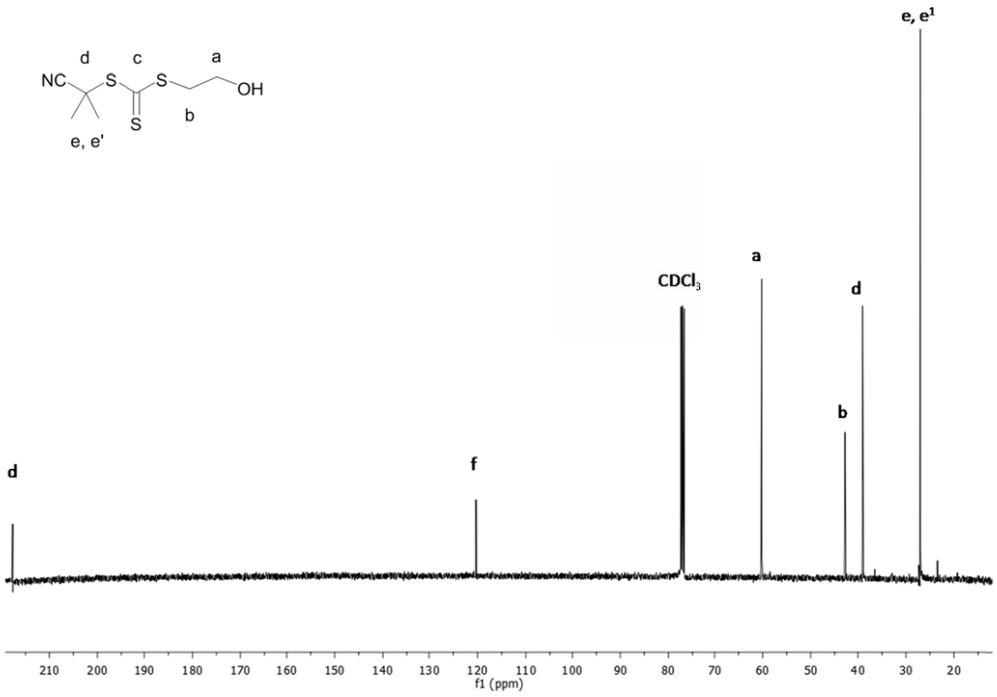
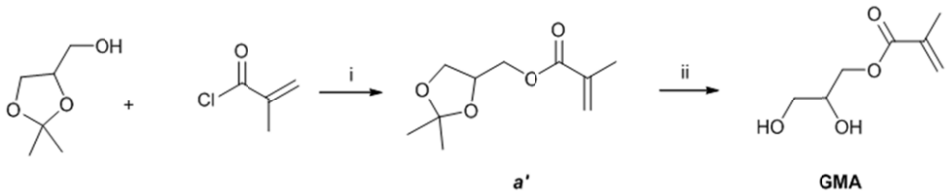


Figure S4. ^{13}C NMR spectrum of purified 2-cyanopropan-2-yl 2-hydroxyethyl carbonotrithioate (CHT) in $CDCl_3$.

4.2 Synthesis of glycerol methacrylate (GMA)



Scheme S2. Synthesis of glycerol methacrylate (GMA). *Reagents and conditions*: (i) Et_3N, Et_2O, 0°C to RT; (ii) THF/(1.0 M HCl_{aq}) 9:1, 48 h.

To a mixture of 1,2-*O*-isopropylidene glycerol (solketal) (20 g, 0.15 mol) and triethylamine (21.2 mL, 0.16 mol) in diethyl ether (80 mL) cooled at 0°C with an ice bath methacryloyl chloride (11 mL, 0.11 mol) was added dropwise over 15 minutes. The reaction was allowed to warm to room temperature and stirred for further 14 hours. $Et_3NH^+Cl^-$ was removed by filtration and the organic layer was washed twice with 100 mL of a saturated sodium chloride aqueous solution, dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting residue (crude solketal

methacrylate (*a'*) was deprotected with a mixture of THF/(1.0 M HCl_{aq}), 9:1 (100 mL). The reaction was monitored by ^1H NMR in DMSO-d_6 and stirred for 48 h until 96% of deprotection was reached. 50 mL of saturated sodium chloride aqueous solution were added to the THF/HCl mixture, the organic layer was separated and the aqueous solution was then washed with DCM (3 x 50 mL). The organic layers were combined and evaporated to dryness under reduced pressure at room temperature to give GMA (21.9 g, 0.136 mol, 91%) as a colourless viscous oil.

^1H NMR (400 MHz, DMSO-d_6): δ = 1.86 (s, 3H, CH_3), 3.39 (m, 2H, -CH_2OH), 3.70 (m, 1H, CHOH), 3.97-4.13 (m, 2H, -CH_2O), 4.65 (t, J = 5.7 Hz, 1H, *H*O-CH-), 4.91 (d, J= 5.3 Hz, 1H, *H*O-CH_2-), 5.67 (q, J = 1.6 Hz, 1H, C*H*$_2$=C), 6.03 (q, J = 1.8 Hz, 1H, C*H*$_2$=C).

$^{13}C\{^1H\}$ NMR (400 MHz, DMSO-d_6): δ = 18.39 (1C, CH_3), 63.02 (1C, CH_2-OH), 66.54 (1C, CH_2-O), 69.63 (1C, CH-OH), 126.25 (1C, *C*H_2=C), 136.41 (1C, CH_2=*C*-CH_3), 167.13 (1C, C-*C*=O).

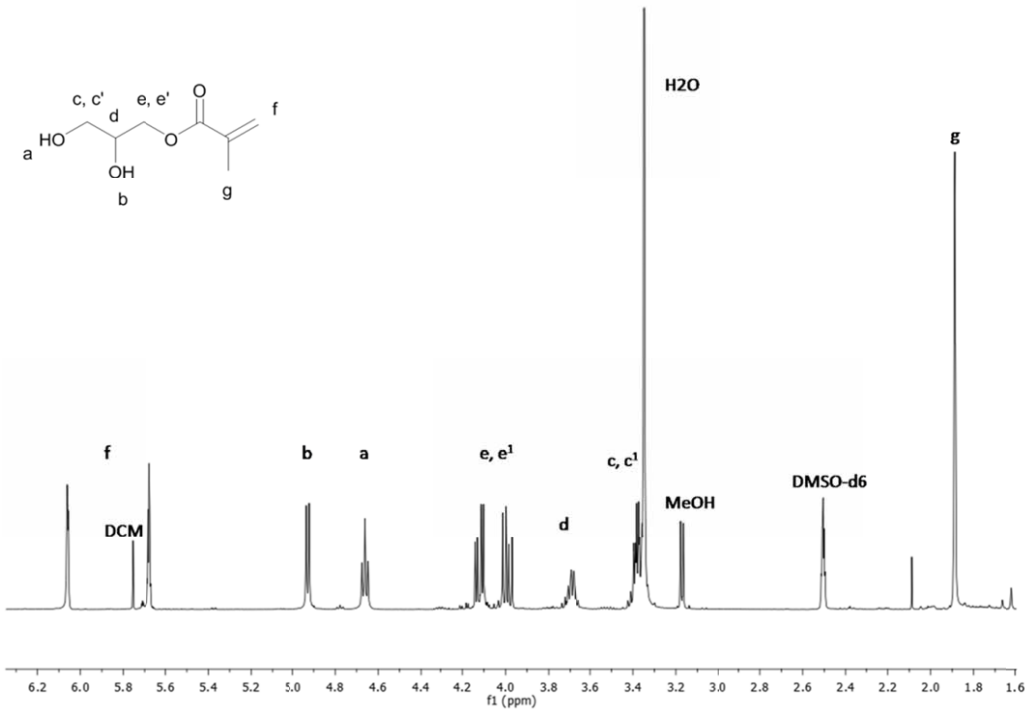


Figure S5. ^1H NMR spectrum of glycerol methacrylate (GMA) in DMSO-d_6.

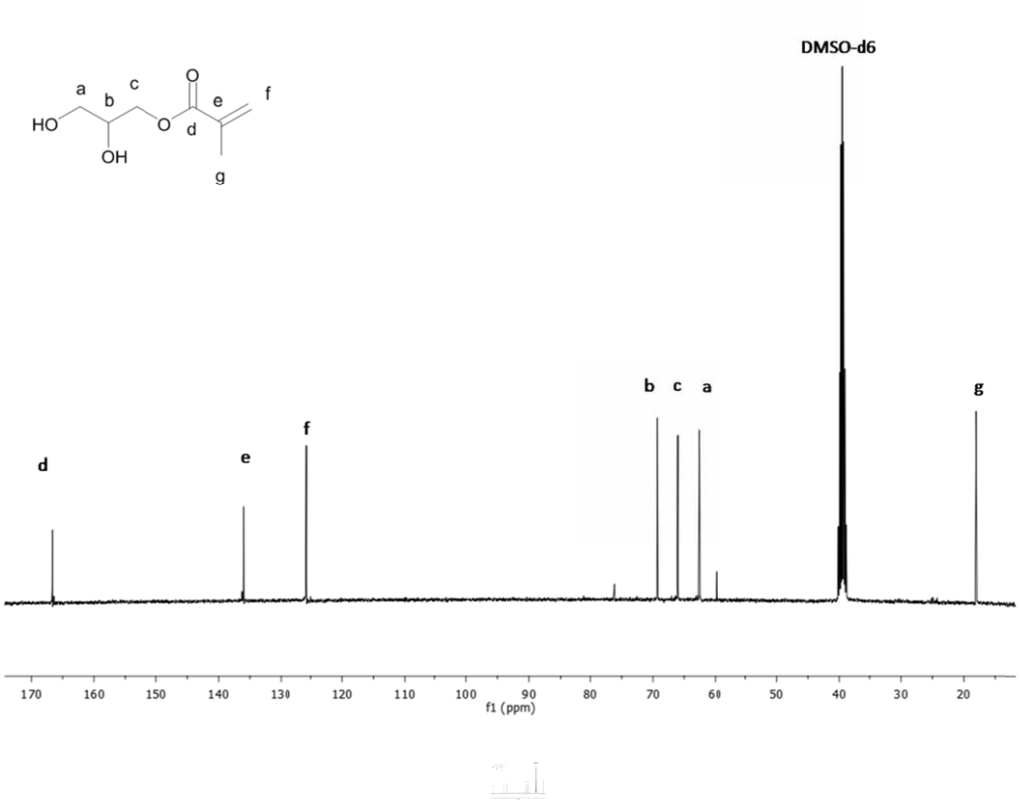
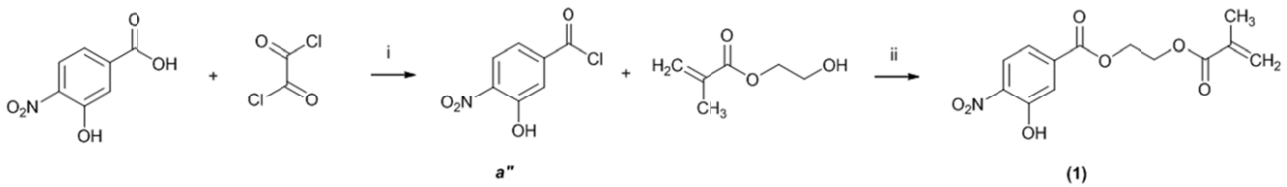


Figure S6. ^{13}C NMR spectrum of GMA in DMSO-d_6.

4.3 Synthesis of 2-(methacryloyloxy)ethyl-3-hydroxy-4-nitrobenzoate (MHN, 1)



Scheme S3. Synthesis of pH responsive monomer 2-(methacryloyloxy)ethyl-3-hydroxy-4-nitrobenzoate (MHN). *Reagent and conditions*: (i) 1. DMF (cat), $CHCl_3$, RT; (ii) Et_3N, DCM, RT.

3-hydroxy-4-nitrobenzoic acid (5.00 g, 27.3 mmol) was suspended in 50 mL of chloroform. Oxalyl chloride (4.15 g, 32.8 mmol) and 1.6 mL of anhydrous DMF were added under stirring. The reaction was allowed to proceed for 1.5 hours. The solvent was removed under reduced pressure; the obtained oil was dissolved in 50 mL of chloroform and the solvent was removed again under

reduced pressure. This procedure was repeated three times to ensure that any traces of residual oxalyl chloride (bp 63-64 °C) were removed from the crude mixture which due to its hydrolytic instability was used for the following step without further purification. A solution of 3-hydroxy-4-nitrobenzoyl chloride (5.0 g, 25 mmol) in anhydrous dichloromethane (30 mL) was added dropwise to a previously prepared mixture of HEMA (13.0 g , 99.6 mmol) and Et_3N (5.2 mL, 38 mmol) in anhydrous dichloromethane (10 mL). The reaction mixture was stirred for 48 hours at ambient temperature. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography (silicagel 60, 35-70 μm) using dichloromethane/petroleum ether 9:1 as the eluent. The solvent in the relevant fractions was evaporated under reduced pressure to give MHN **(1)** (5.80 g, 19.7 mmol, 72.2 %) as a yellow solid.

ESI-TOF mass spectrometry: expected m/z for $[M-H]^-$ 294.07, found 294.03. FT-IR: ν 3434, 2092, 1628, 1537, 1480, 1444, 1218, 1075, 946, 745 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.95 (m, 3H, CH_3), 4.48-4.62 (m, 4H, CH_2), 5.60 (m, 1H, C=CH*H*), 6.13 (s, 1H, C=C*H*H), 7.61 (dd, *J* = 8.8, 1.8 Hz, 1H, CH aromatic), 7.82 (d, *J*= 1.8 Hz, 1H, CH aromatic), 8.17 (d, *J* = 8.8 Hz, 1H, CH aromatic). 10.49 (s, 1H, O*H*). $^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$, δ, ppm): 18.40 (1C, CH_3), 62.20 (1C, CH_2O), 63.80 (1C, CH_2O), 120.77 (1C, C aromatic, CH), 121.91 (1C, CH), 125.47 (1C, C aromatic), 126.41 (1C, CH_2), 135.95 (1C, C aromatic), 136.03 (1C, *C*-CH_3), 137.79 (1C, C aromatic), 154.78 (1C, C aromatic, C-OH), 164.23 (1C, C=O), 167.19 (1C, C=O).

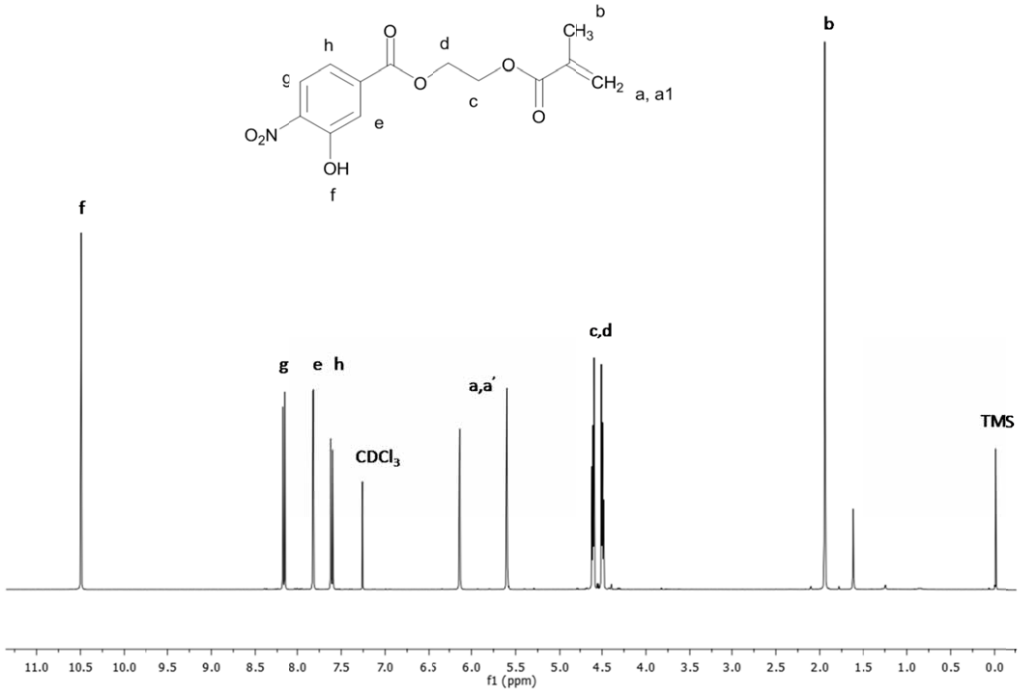


Figure S7. ^{1}H NMR spectrum of purified MHN **(1)** in $CDCl_3$.

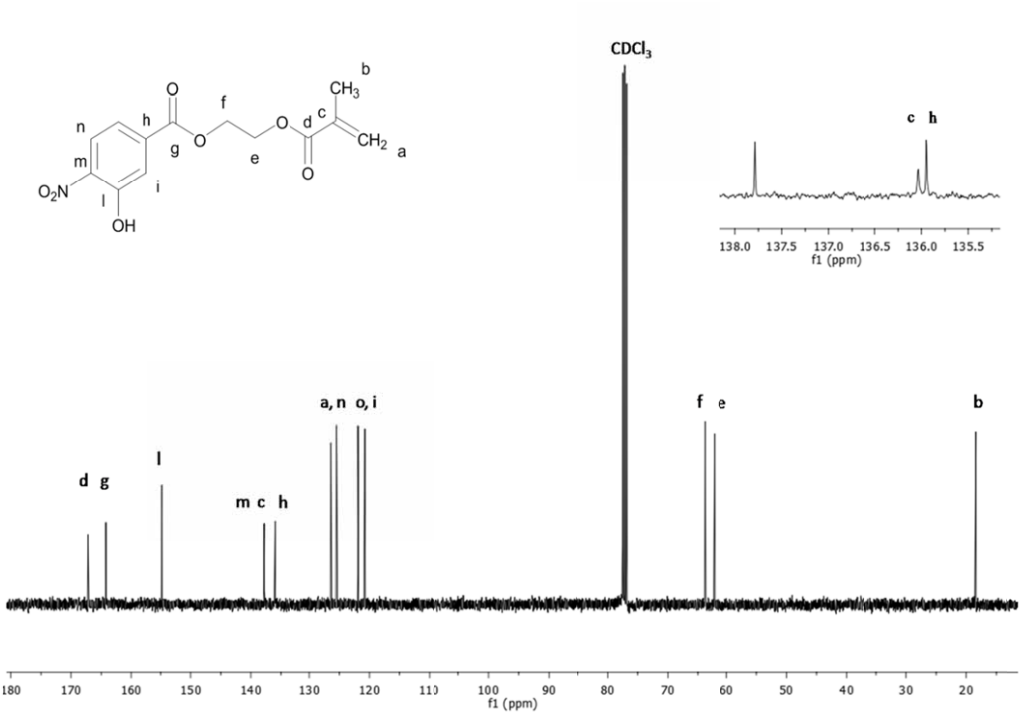


Figure S8. ^{13}C NMR spectrum of purified MHN **(1)** in $CDCl_3$.

4.3 Synthesis of 2-(methacryloyloxy)ethyl-3-chloro-4-hydroxybenzoate MCH (2), 2-(methacryloyloxy)ethyl-3,5-dichloro-4-hydroxybenzoate MDCH (3) and 2-(methacryloyloxy)ethyl-3-chloro-4-methoxybenzoate (MCM, 4)

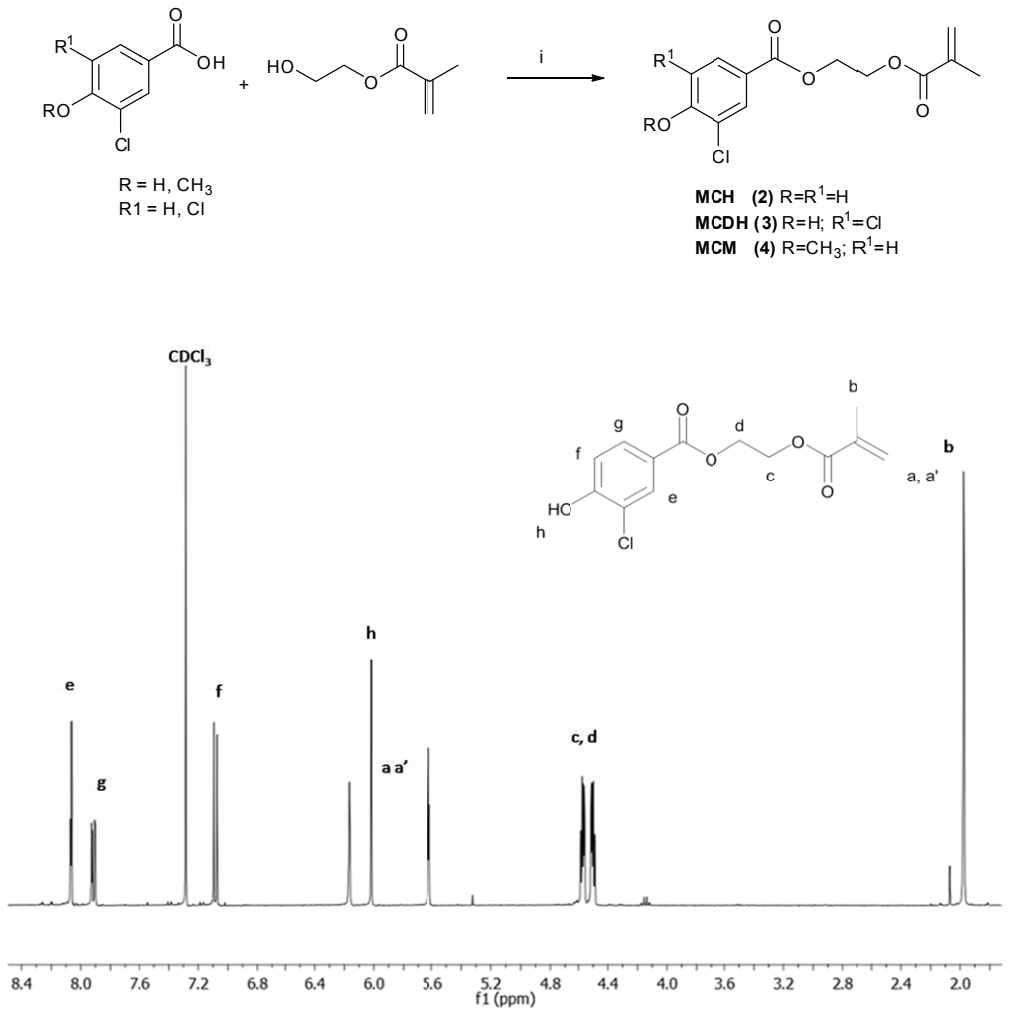


Figure S9. ^{1}H NMR spectrum of purified MCH **(2)** in $CDCl_3$.

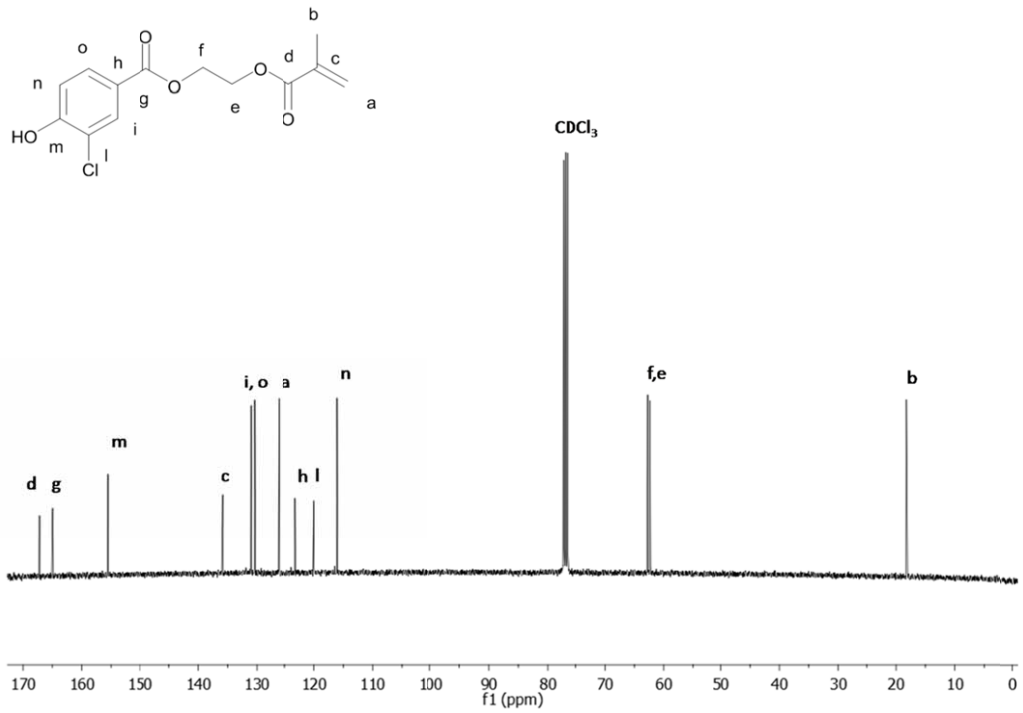


Figure S10. ^{13}C NMR spectrum of purified MCH **(2)** in $CDCl_3$

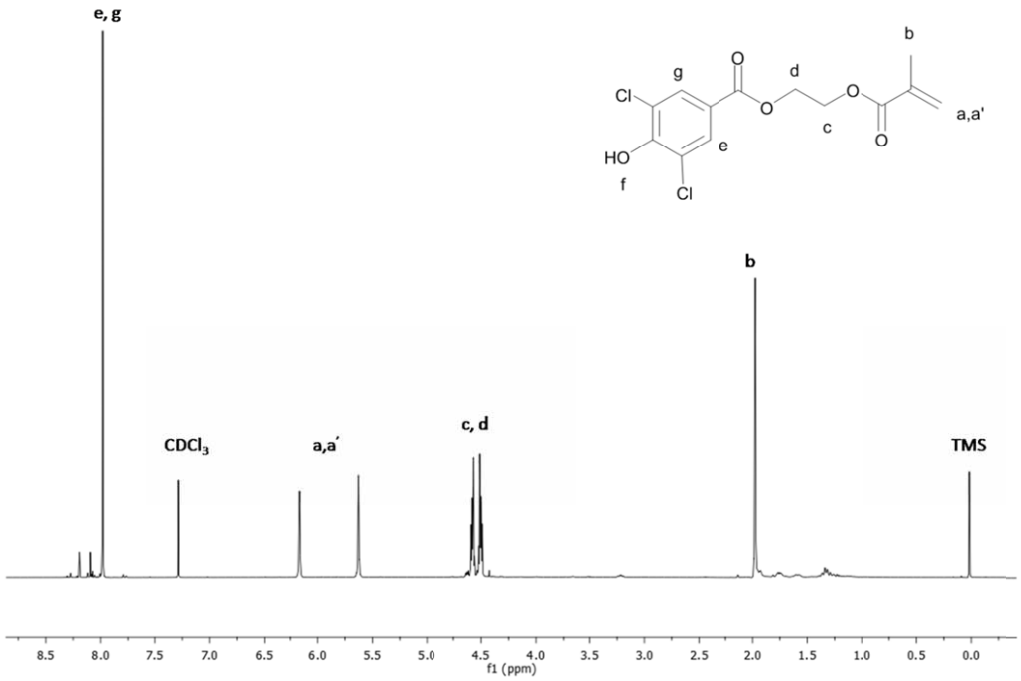


Figure S11. ^{1}H NMR spectrum of purified MDCH **(3)** in $CDCl_3$.

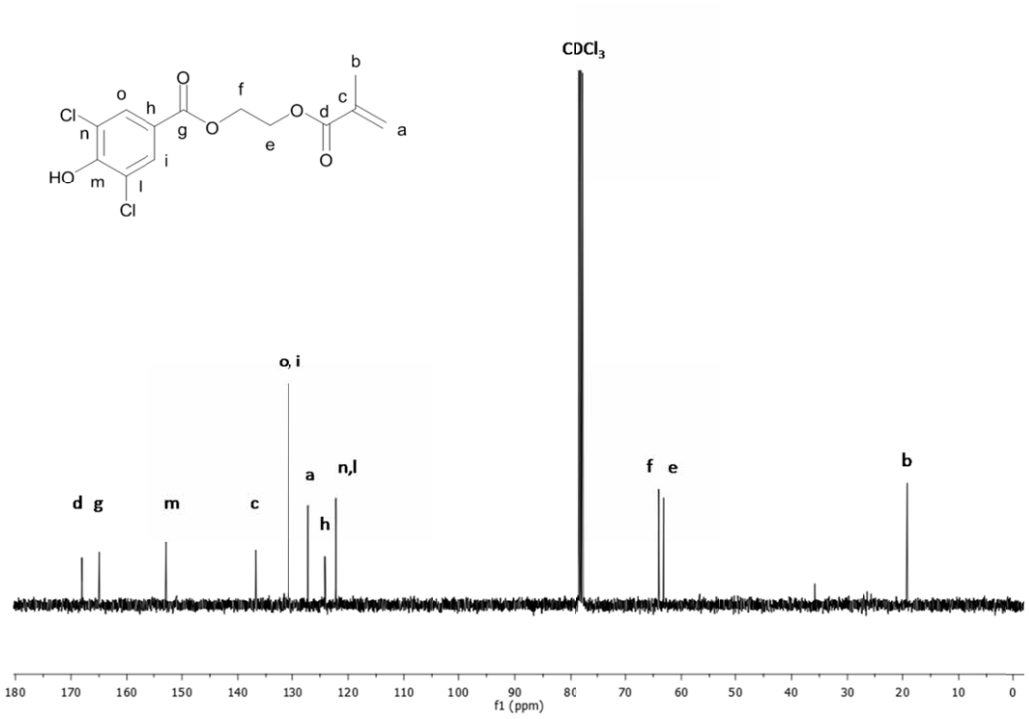


Figure S12. ^{13}C NMR spectrum of purified MDCH **(3)** in $CDCl_3$.

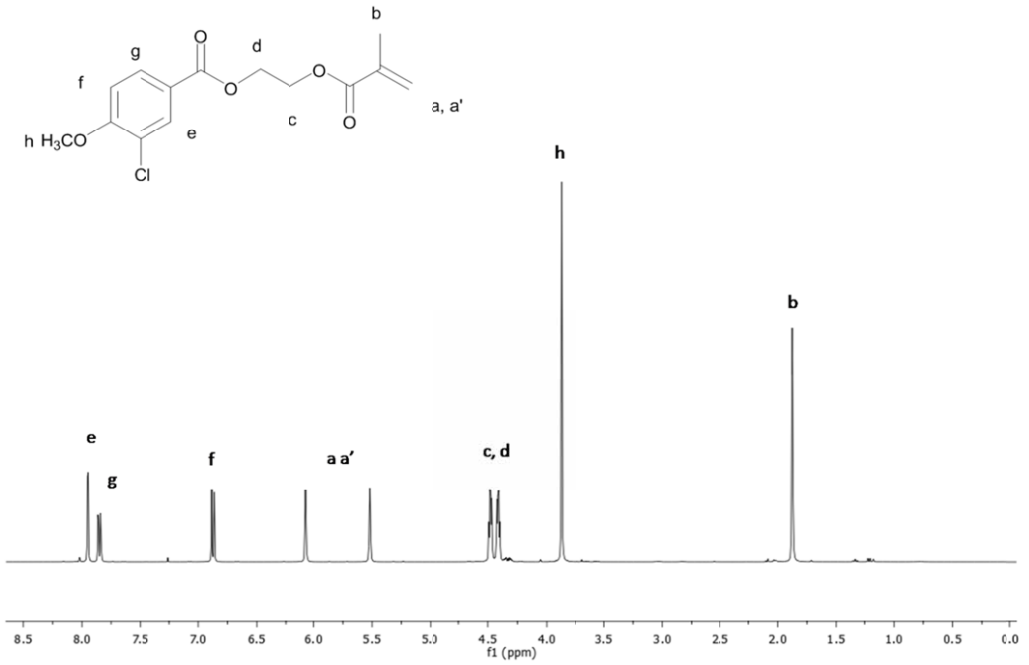


Figure S13. ^{1}H NMR spectrum of purified control monomer MCM **(4)** in $CDCl_3$.

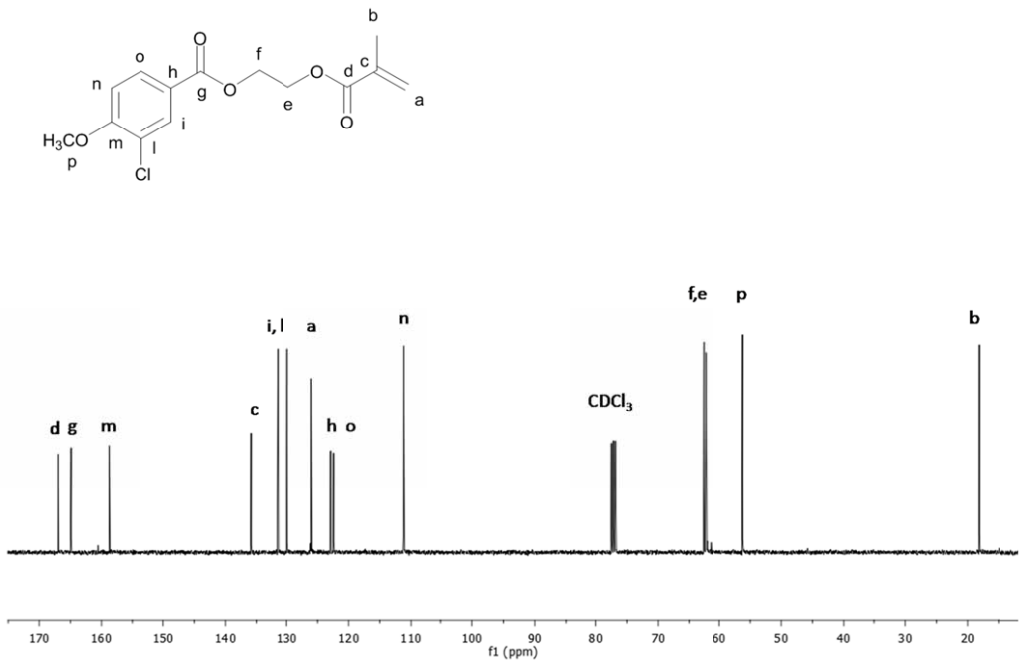
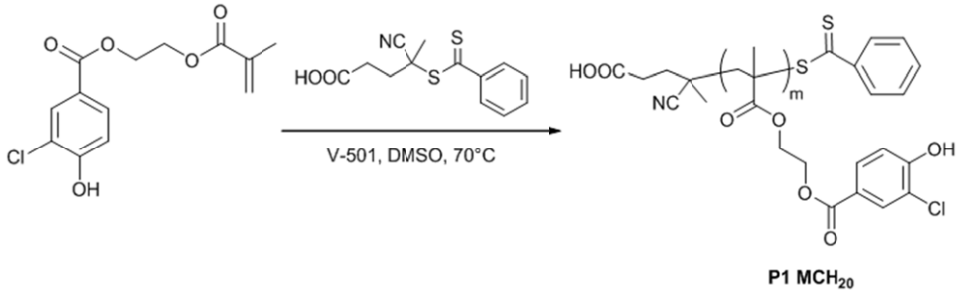


Figure S14. ^{13}C NMR spectrum of purified control monomer MCM **(4)** in $CDCl_3$.

4.4 Synthesis of the homopolymer MCH_{20} (P1)



Scheme S4: Synthesis of MCH_{20} homopolymer **P1**.

A solution of MCH (292 mg, 1.03 mmol) in 700 µL of anhydrous DMSO was placed in a Schlenk tube equipped with a magnetic bar. V-501 (2.4 mg, 8.6 µmol, 240 µL of 10 mg mL^{-1} stock solution in DMSO) and 4-cyano-4-((thiobenzoyl)sulfanyl)pentanoic acid (12 mg, 41 µmol, 120 µL of 100 mg mL^{-1} stock solution in DMSO) were added to the MCH solution. The reaction mixture was degassed by 3 freeze-pump-thaw cycles and the polymerization reaction was started placing the

tube in an oil bath pre-heated at 70°C. The polymerization was monitored by ^{1}H NMR analysis in DMSO-d_6 of aliquots withdrawn at regular intervals of time. When the desired conversion of 80% was reached the polymerization was stopped by lifting the reactor from the oil bath and exposing the reaction mixture to air. The polymer was precipitated in diethyl ether at 0°C, redissolved in dichloromethane and precipitated again diethyl ether at 0°C to remove residual traces of DMF. After filtration and removal of residual diethyl ether under reduced pressure **P1** (146 mg) was isolated as a pink solid. $M_{n,theor}$ = 6.0 kDa; $M_{n,GPC}$ = 14.9 kDa, PDI = 1.16.

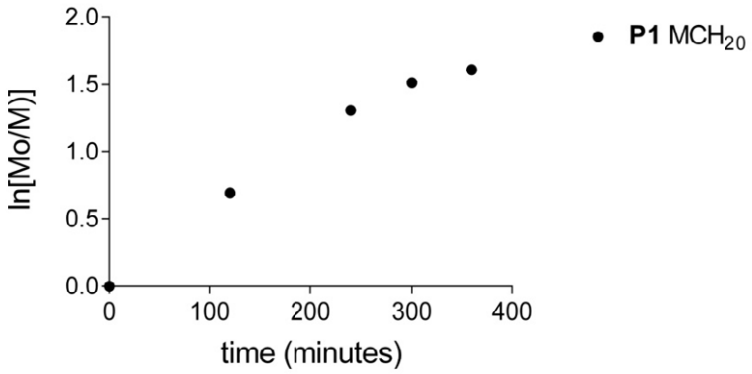


Figure S15. First order kinetic plot for the homopolymerisation of MCH in DMSO. [MCH]:[CTA]:[AIBN] = 25: 1:0.2.

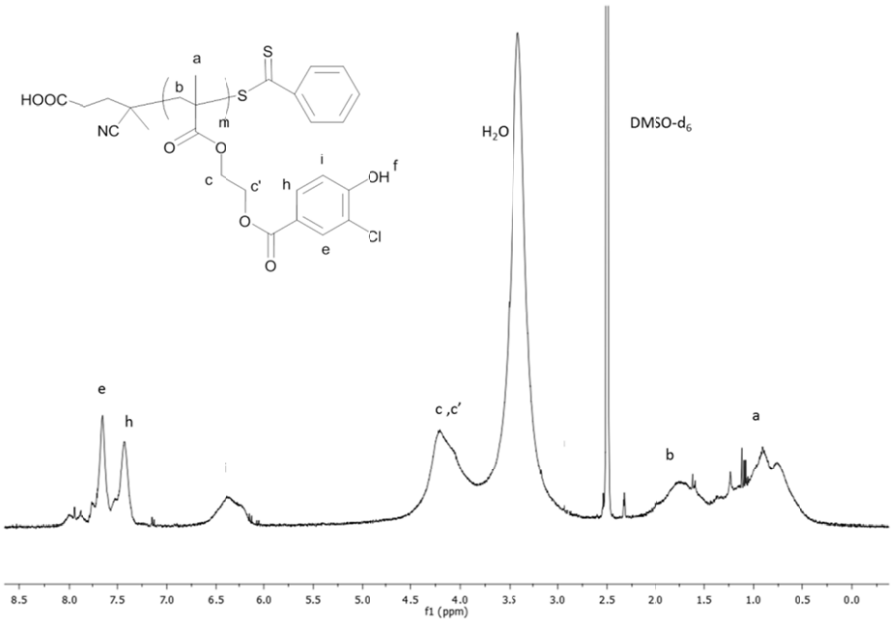
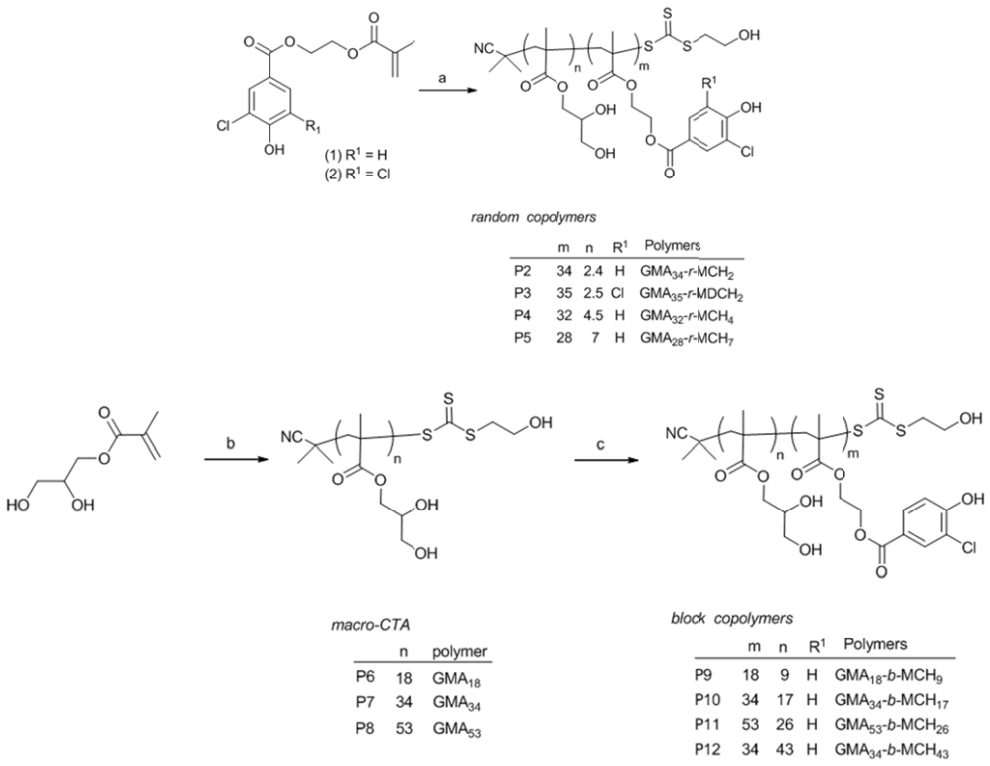


Figure S16. ^{1}H NMR spectrum of MCH_{20} **P1** in DMSO-d_6.

4.5 Synthesis poly(GMA-*r*-MHC) and poly(GMA-*b*-MDHC) random and block copolymers.



Scheme S5. *Reagents and conditions*: (a) CHT, AIBN, GMA, DMF, 70°C; (b) CHT, AIBN, DMF 70°C; (c) MCH, AIBN, DMF, 70°C.

Four poly(GMA-*r*-MHC) random copolymers were synthesized using an increasing MCH/GMA molar ratio (Table S2) (MDCH/GMA feed ratio of 1:15 for GMA_{35}-*r*-$MDCH_2$ **P3)** and a monomers/RAFT agent constant ratio of 1:50.

Table S2. Composition, conversion, yield, M_n and PDi of poly(GMA-*co*-MCH) and poly(GMA-*co*-MCM) random copolymers.

Code	Polymers	Feed m.r. MCH/GMA	m.r MCH/GMA[a]	Conversion[b]	Yield[c]	$M_{n,theor}$	PDI[d]
P2	GMA_{34}-*r*-MCH_2	1:15	1:14	76%	61%	6.3	1.27
P3	GMA_{35}-*r*-$MDCH_2$	1:15	1:14	78%	50%	6.5	1.16
P4	GMA_{32}-*r*-MCH_4	1:8	1:7	72%	68%	6.5	1.26
P5	GMA_{28}-*r*-MCH_7	1:4	1:4	70%	79%	6.8	1.24

[a]Estimated by ^{1}H NMR in DMSO-d_6, [b]total conversion (MCH+GMA); [c]yield of the purification steps (recovery yield, %); [d]polydispersity index (PDI) of the synthesized polymers as determined by SEC using DMF + 0.1% w/w LiBr as the mobile phase in a system calibrated with PMMA standards.

Typical polymerization conditions (also reported in the manuscript): synthesis of GMA_{28}-*r*-MCH_7 P5. GMA (1.57 g, 9.80 mmol), MHC (700 mg, 2.46 mmol), CHT (54 mg, 0.25 mmol) and AIBN (20 mg, 0.12 mmol) were dissolved in anhydrous DMF (10 mL) in a Schlenk tube equipped with a magnetic follower. The tube was sealed with a rubber septum, the reaction mixture was degassed by three freeze-pump-thaw cycles and the reaction was started by placing the tube in a pre-heated oil bath at 70°C. The polymerization was monitored by ^{1}H NMR analysis in DMSO-d_6 of aliquots withdrawn at regular intervals of time. At 72% conversion the polymerization was stopped by lifting the reactor from the oil bath and exposing the reaction solution to air. The polymer was precipitated in diethyl ether at 0°C, re-dissolved in methanol and precipitated again to remove residual traces of DMF. After filtration, residual Et_2O was removed under reduced pressure to give **P5** (1.3 g) as a yellow solid. $M_{n,theor}$ = 6.8 kDa; M_n (GPC) = 14.3 kDa, PDi = 1.24.

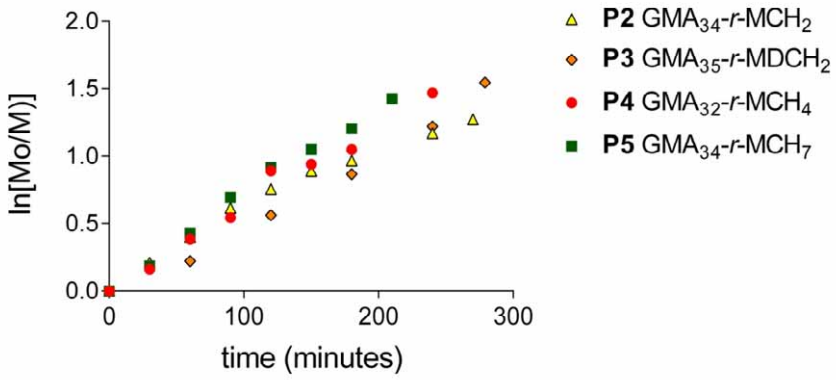


Figure S17. First order kinetic plots for the synthesis of random copolymers **P2** (▲), **P3** (♦), **P4** (●) and **P5** (■). The progression of the polymerizations were monitored by ^{1}H NMR analysis in DMSO-d_6 of samples withdrawn at scheduled times.

All polymerisations showed an increase of M_n with the conversion, below a conversion vs. M_n is reported as an example.

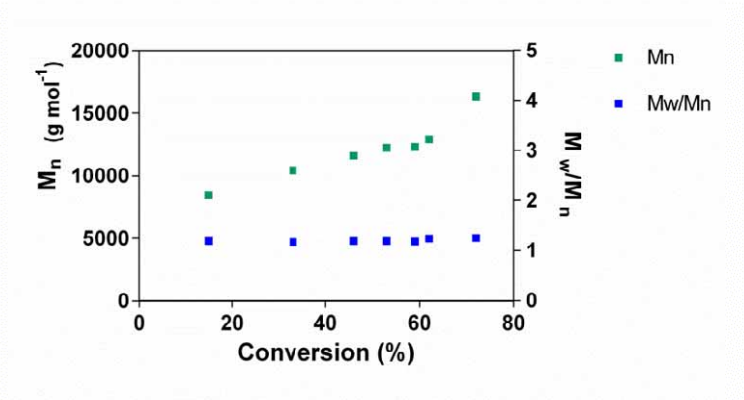


Figure S18. Synthesis of GMA_{34}-*r*-MCH_2 **P2**: conversion vs. M_n, and conversion vs. M_w/M_n plots. Reaction conditions: [40]:[10]:[1]:[0.5], DMF, 70°C.

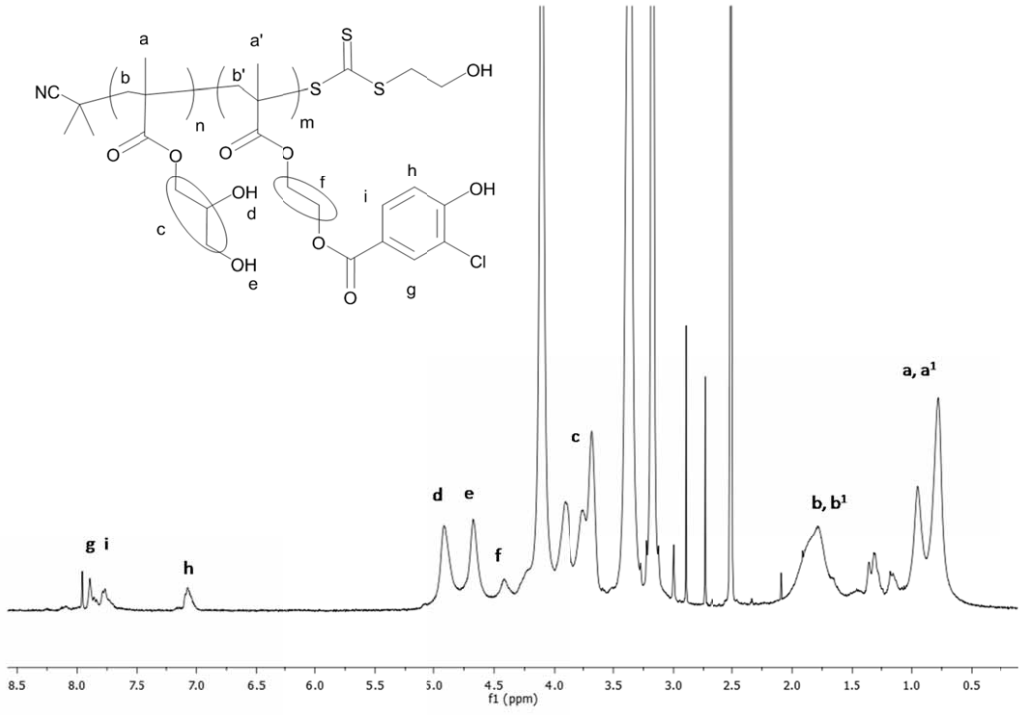


Figure S19. ^{1}H NMR spectrum of GMA_{32}-*r*-MCH_4 **P5** random copolymer in DMSO-d_6.

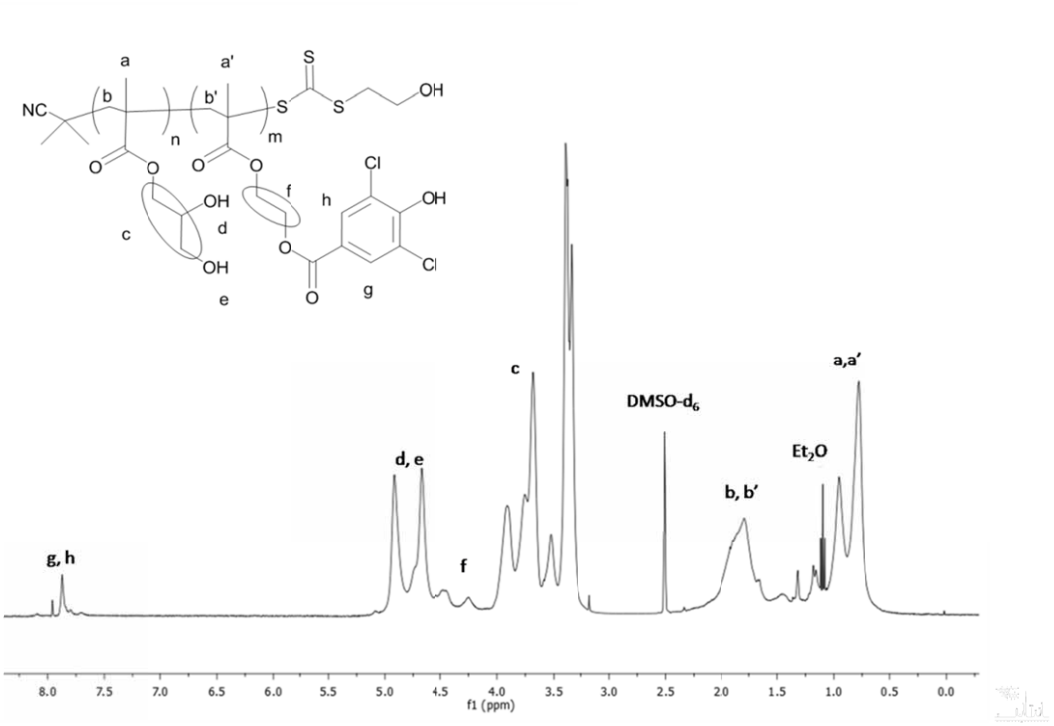


Figure S20. ^{1}H NMR spectrum of GMA_{35}-*r*-$MDCH_2$ **P3** random copolymer in DMSO-d_6.

The synthesis of the block copolymers poly(GMA-*b*-MCH) was performed following the same polymerization conditions reported for the random copolymers describe in "**T**ypical polymerization conditions: synthesis of GMA_{32}-*r*-MCH_4 **P5**". GMA was polymerized via RAFT, purified and used as macro-chain transfer agent (macro-CTA**)** to grow the pH-responsive MCH block. Three pGMA macro-transfer agents (**P6**, **P7**, **P8**) were synthesized using a constant [AIBN]/[CHT] molar ratio of 0.5:1. [CHT]/[GMA] feed ratios were varied as reported in Table S3.

Table S3. [CHT RAFT Agent]/[GMA] molar ratio, polymer compositions, conversion, yield, M_n and PDi of pGMA macro-CTA.

Code	Polymers	Feed CHT/GMA	CHT/GMA$_{theor}$ [a]	Conversion	$M_{n,theor}$ (kDa)	PDI [b]
P6	GMA_{18}	1:20	1:18	87%	3.0	1.28
P7	GMA_{34}	1:40	1:34	84%	5.7	1.26
P8	GMA_{53}	1:60	1:53	88%	8.7	n.d.

[a]Determined by ^{1}H NMR in DMSO-d_6,; [b]Polydispersity index (PDI) of the synthesized polymers as determined by SEC using DMF + 0.1% LiBr as the mobile phase in a system calibrated with PMMA standards.

Typical polymerization conditions: synthesis of GMA_{18} P6. GMA (2.00 g, 12.5 mmol), CHT (138 mg, 0.620 mmol) and AIBN (51 mg, 0.31 mmol) were dissolved in anhydrous DMF (12 mL) in a Schlenk tube equipped with a magnetic follower. The tube was sealed with a rubber septum, the reaction mixture was degassed by three freeze-pump-thaw cycles and the reaction was started by placing the tube in a pre-heated oil bath at 70°C. The polymerization was monitored by ^{1}H NMR analysis in DMSO-d_6 of aliquots withdrawn at regular intervals of time. At 87% conversion the polymerization was stopped by lifting the reactor from the oil bath and exposing the reaction solution to air. The polymer was precipitated in a mixture of petroleum ether/diethyl ether 1:1 at 0°C, re-dissolved in methanol and precipitated again to remove residual traces of DMF. After filtration, residual Et_2O was removed under reduced pressure to give **P6** (1.3 g) as a yellow solid. $M_{n,theor}$ = 3.0 kDa; M_n (GPC) = 8.4 kDa, PDi = 1.28.

The pGMA macro-CTAs were then used to prepare the block copolymers **P9-P12** with the pH-responsive monomer MCH **(2)**. The feed ratios and the main characteristics of the poly(GMA-*b*-MCH) block copolymers are reported in table S4.

Table S4. Monomers feed ratios, conversions, M_n and PDi of poly(GMA-*b*-MCH) **P9-P12** block copolymers.

Code	Polymers	Macro-CTA	feed GMA/MHC	GMA/MCH[a]	Conversion	$M_{n,theor,}$ (kDa)	PDI[b]
P9	GMA_{18}-*b*- MCH_9	**P6**	1.8:1	2:1	88%	5.3	1.30
P10	GMA_{34}-*b*- MCH_{17}	**P7**	1.8:1	2:1	89%	10.4	1.28
P11	GMA_{53}-*b*- MCH_{26}	**P8**	1.8:1	2:1	87%	16.1	1.39
P12	GMA_{34}-*b*- MCH_{43}	**P7**	1:1.46	1:1.25	88%	18.0	1.30

[a]Estimated by ^{1}H NMR in DMSO-d_6, [b]Polydispersity index (PDI) of the synthesized polymers determined by SEC using DMF + 0.1% LiBr as the mobile phase in a system calibrated with PMMA standards.

All the macro-transfer agent and block copolymers synthesized were characterized by GPC and ^{1}H NMR in DMSO-d_6 (^{1}H NMR of **P6** and **P11** are reported here as an example).

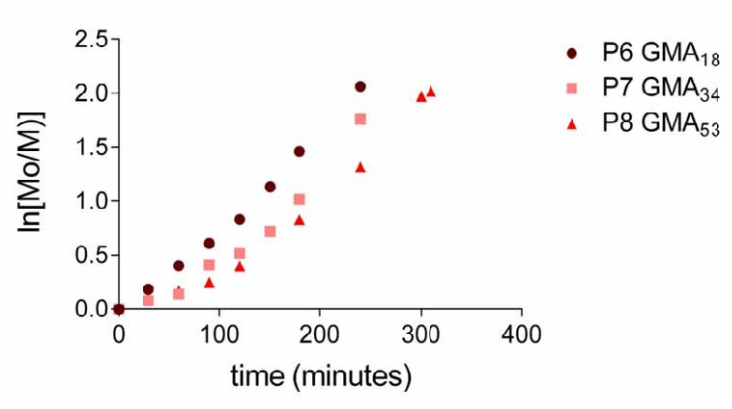


Figure S21. First order kinetic plots for the synthesis of pGMA macro-CTA **P6** (●), **P7** (■) and **P8** (▲). The progression of the polymerizations was monitored by ^{1}H NMR in DMSO-d_6.

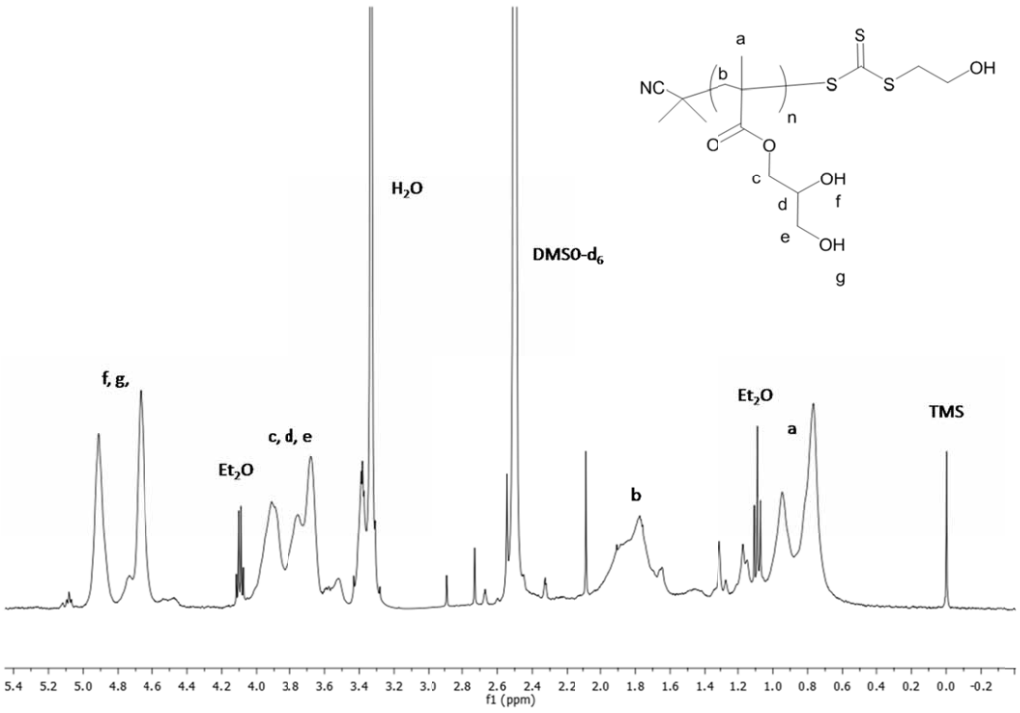


Figure S22. ^{1}H NMR spectrum of purified GMA_{18} **P6** macro-CTA in DMSO-d_6.

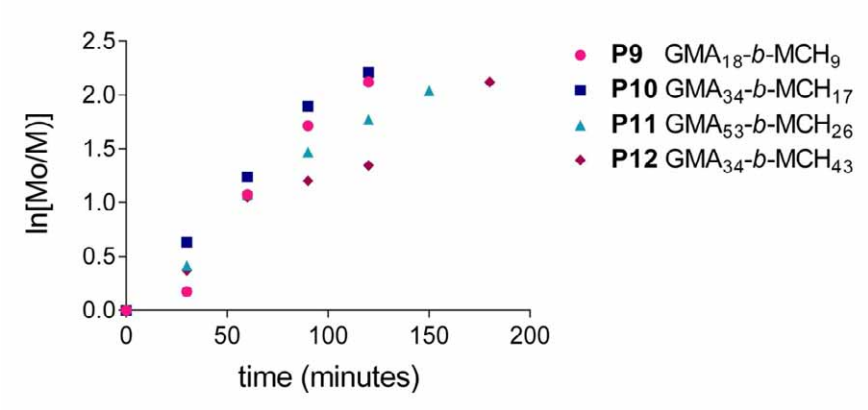


Figure S23. First order kinetic plots for the synthesis of poly(GMA-*b*-MCH) block copolymers **P9** (●), **P10** (■), **P11** (▲) and **P12** (♦). The progression of the polymerizations was monitored by ^{1}H NMR in DMSO-d_6.

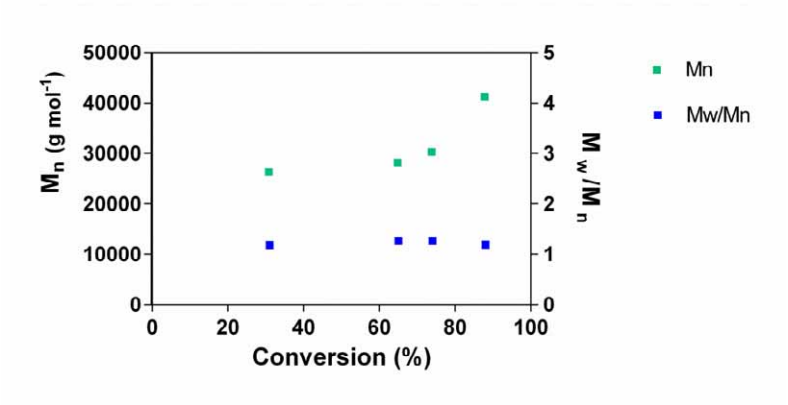


Figure S24. Synthesis of GMA_{34}-*b*- MCH_{43} **P12**: conversion vs. M_n, and conversion vs. M_w/M_n plots.

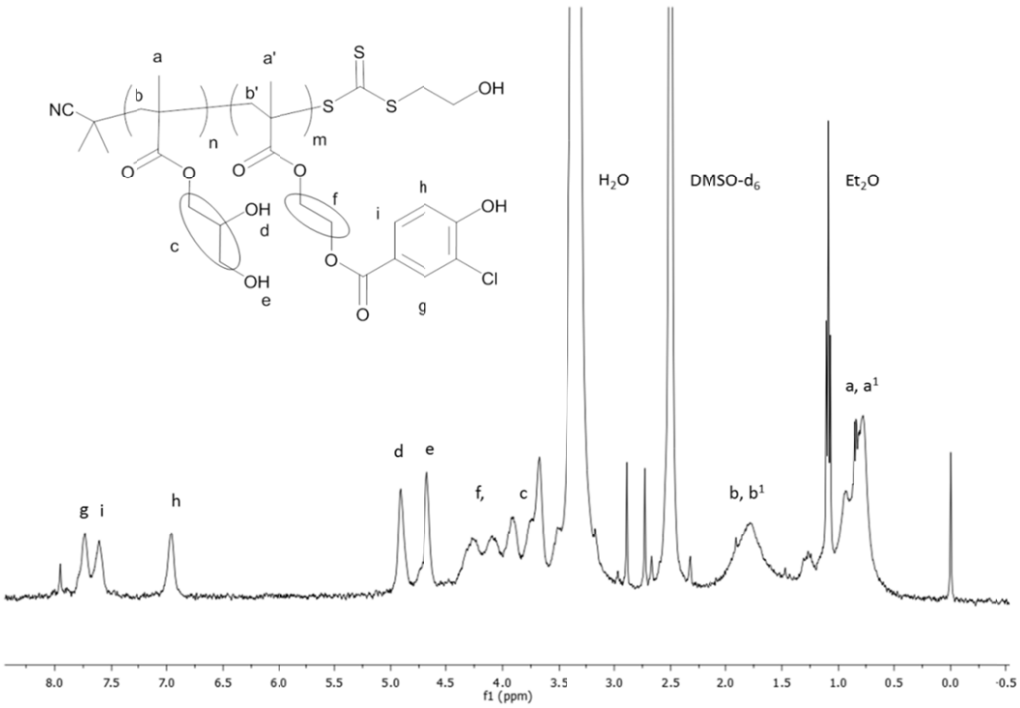
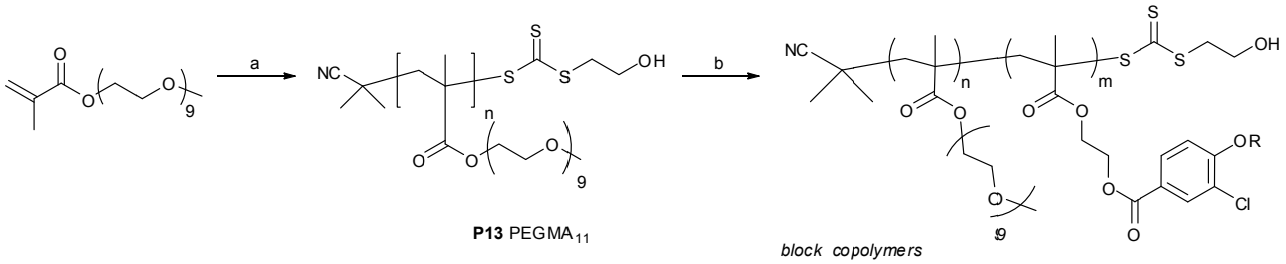


Figure S25. ^{1}H NMR spectrum of purified GMA_{53}-*b*-MCH_{26} **P11** block copolymer in DMSO-d_6.

4.6 Synthesis of $PEGMA_{11}$ macro-CTA (P13) and block copolymers p(PEGMA-*b*-MHC) (P14 and P15) and poly(PEGMA-*b*-MCM) (P16)



	n	m	R	Polymers
P14	11	21	H	$PEGMA_{11}$-*r*-MCH_{21}
P15	11	38	H	$PEGMA_{11}$-*r*-MCH_{38}
P16	11	20	CH_3	$PEGMA_{11}$-*r*-MCM_{20}

Scheme S6. Synthesis of $PEGMA_{11}$ macro-CTA and pPEGMA-based block copolymers with MCH and MCM. *Reagents and conditions*: (a) CHT, AIBN, THF 65°C; (b) AIBN, MCM or MCH, DMF 70°C.

The synthesis of the PEGMA di-block copolymers was carried out by following a two-step protocol. In the first step, $PEGMA_{11}$ was prepared by RAFT polymerization using a 1:15 [CHT]/[$mPEGMA_{475}$] molar ratio (Scheme S6, step a).

$mPEGMA_{475}$ (9.25 mL, 21.0 mmol), CHT (310 mg, 1.40 mmol) and AIBN (115 mg, 0.700 mmol) were dissolved in 20 mL of anhydrous THF and placed in a Schlenk tube equipped with a magnetic stirrer. The reaction mixture was sealed with a rubber septum and degassed by three freeze-pump-thaw cycles. The tube was immersed into a pre-heated oil bath at 65°C and the polymerization reached 73% conversion in 3 hours. The reaction was stopped by opening the tube to air and cooling to ambient temperature and the polymer was precipitated twice in a 1:1 (vol/vol) petroleum ether/diethyl ether mixture cooled to 0°C to give $PEGMA_{11}$ (7.0 g) macro transfer agent as a yellow viscous oil. $M_{n,theor}$ = 5.5 kDa; M_n GPC = 10.2 kDa, PDI = 1.12.

An approximate M_n for $PEGMA_{11}$ macro-CTA was also estimated by UV-vis analysis, using the molar extinction coefficient of CHT in $CHCl_3$ at 440 nm, which was found to be 30.46 L mol^{-1} cm^{-1}[1]. It is important to stress here that this approach suffers from two important approximations:

i) that no loss of trithiocarbonate chain-ends via either irreversible chain termination or hydrolysis occurred. The latter is rather unlikely under the reaction conditions employed here. RAFT polymerisation is routinely carried out under aqueous conditions, here hydrolysis due to traces of water in the solvent or PEGMA are, as mentioned above, unlikely especially when relatively hydrolytically stable trithiocarbonates are used as the transfer agent. However, RAFT polymerisation is a radical process and irreversible termination side-reactions can be minimised, but not entirely suppressed.

ii) that the molar extinction coefficient of the trithiocarbonate chromophore does not change substantially by going from CHT to $PEGMA_{11}$ **P13**. Although this cannot be proved experimentally, on paper this approximation could be acceptable.

Despite the intrinsic limitations of this approach this experiment was carried out, with the aim of obtaining an approximate estimate of the extent of the irreversible termination events. Using 100 mg mL^{-1} solutions of $PEGMA_{11}$ in $CHCl_3$, an approximate molecular weight of 6.3 kDa could be estimated, which, when compared to the theoretical number average molecular weight of 5.5 kDa, confirmed that irreversible termination, if present, was minimal.

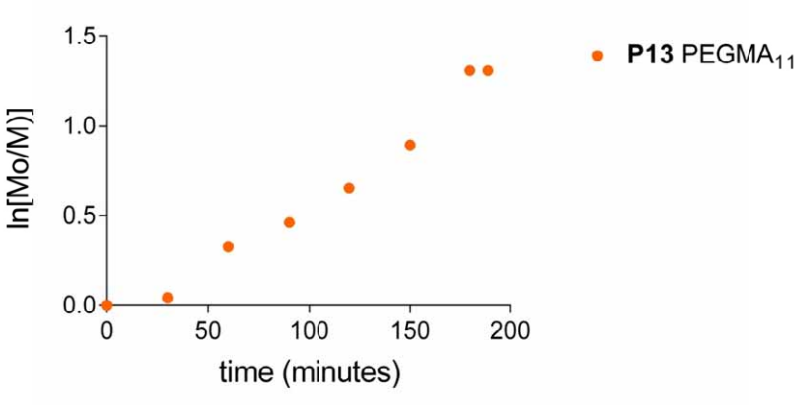


Figure S26. First order kinetic plot for the synthesis of $PEGMA_{11}$ **P13**.

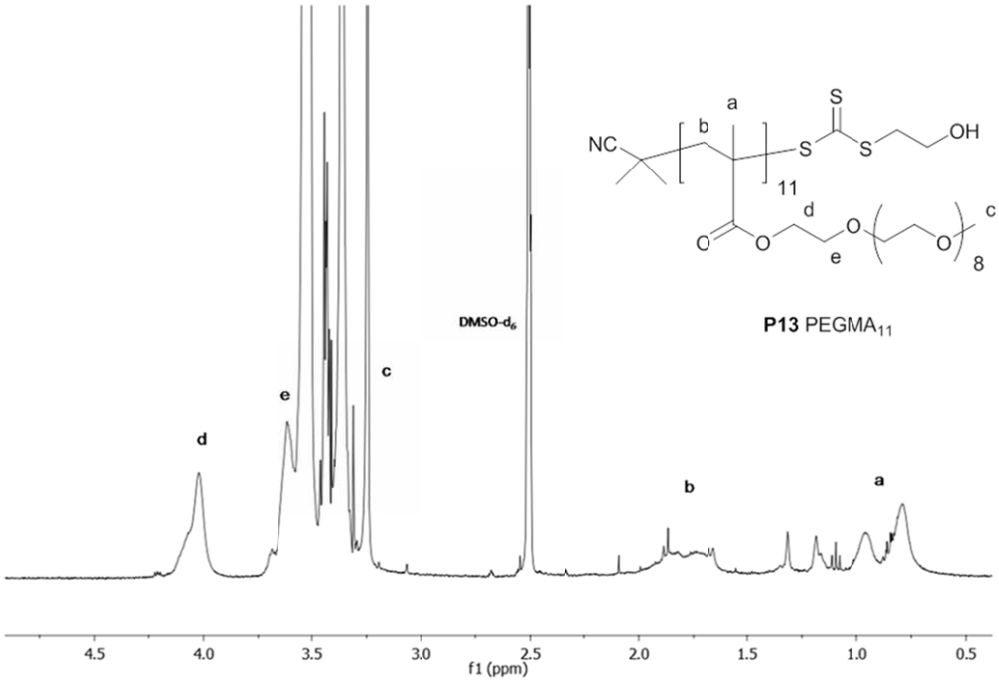


Figure S27. ^1H NMR spectrum of $PEGMA_{11}$ **P13** macro-CTA in DMSO-d_6.

In the second step, $PEGMA_{11}$ **P13** was used as macro-CTA for the addition of the pH responsive block (pMCH) or the pH unsensitive control block (pMCM) to give the polymers **P14**, **P15**, and **P16**. The monomer feed ratios and the main features of the resulting block copolymers are described in Table S5.

Table S5. [$mPEGMA_{475}$]/[MCH] or [MCM] feed ratios, conversions, yield, theoretical M_n and PDI.

Code	Polymer[a]	feed ratios $mPEGMA_{475}$/MCH (or MCM)	Conversion	$M_{n\ theor}$ (kDa)	PDI[b]
P13	$PEGMA_{11}$	1:15[c]	73%	5.5	1.16
P14	$PEGMA_{11}$-*b*-MCH_{21}	11:26	82%	11.5	1.10
P15	$PEGMA_{11}$-*b*-MCH_{38}	11:47	80%	16.3	1.15
P16	$PEGMA_{11}$-*b*-MCM_{20}	11:30	65%	11.5	1.14

[a]PEGMA:MCH and PEGMA:MCM ratios in the block copolymers were determined ^{1}H NMR in DMSO-d_6, [b]Polydispersity index (PDI) of the synthesized polymers determined by SEC using DMF + 0.1% w/w LiBr as the mobile phase in a system calibrated with PMMA standards. [c]for **P13** the feed ratio is for CTA / $mPEGMA_{475}$

Typical polymerization conditions: synthesis of $PEGMA_{11}$-*b*-MCH_{21} P14. $PEGMA_{11}$ **P13** (1.0 g, 0.18 mmol), MCH (1.33 g, 4.68 mmol) and AIBN (14 mg, 0.084 mmol) were dissolved in 4 mL of anhydrous DMF in a Schlenk tube equipped with a magnetic follower. The tube was sealed with a rubber septum, the reaction mixture was degassed by three cycles of freeze-pump-thaw and the reaction was started immersing the tube in a pre-heated oil bath at 70°C. The polymerization was monitored by ^{1}H NMR analysis in DMSO-d_6 of aliquots withdrawn at regular intervals of time. At 82% conversion the polymerization was stopped by lifting the reactor from the oil bath and exposing the reaction solution to air. The polymer was precipitated in diethyl ether at 0°C, redissolved in dichloromethane and precipitated again diethyl ether at 0°C to remove residual traces of DMF. After filtration, residual Et_2O was removed under reduced pressure to give **P14** (1.07 g) as a yellow viscous oil. $M_{n\ theor}$= 11.8 g mol^{-1}; $M_{n,\ GPC}$ = 22.8 kDa, PDI = 1.10.

^{1}H NMR spectra of purified **P14** and **P16** in DMSO-d_6 are shown in Figure S29 and S30, respectively.

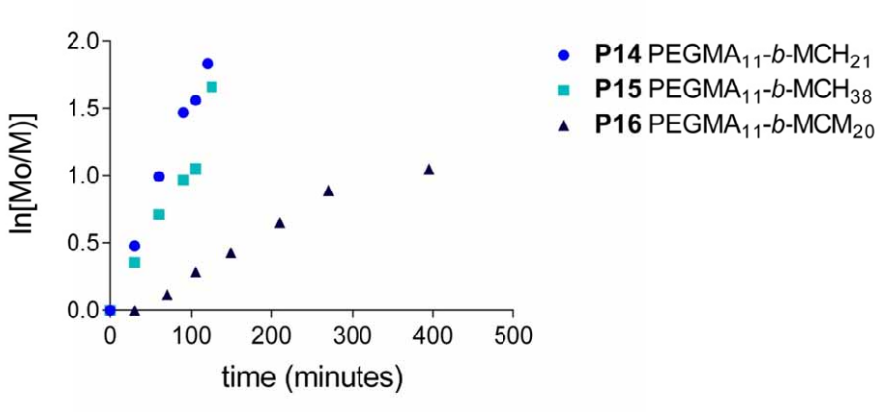


Figure S28. First order kinetic plots for the synthesis of PEGMA$_{11}$-*b*-MCH$_{21}$ **P14** (●), PEGMA$_{11}$-*b*-MCH$_{38}$ **P15** (■) and (▲) PEGMA$_{11}$-*b*-MCM$_{20}$ **P16**.

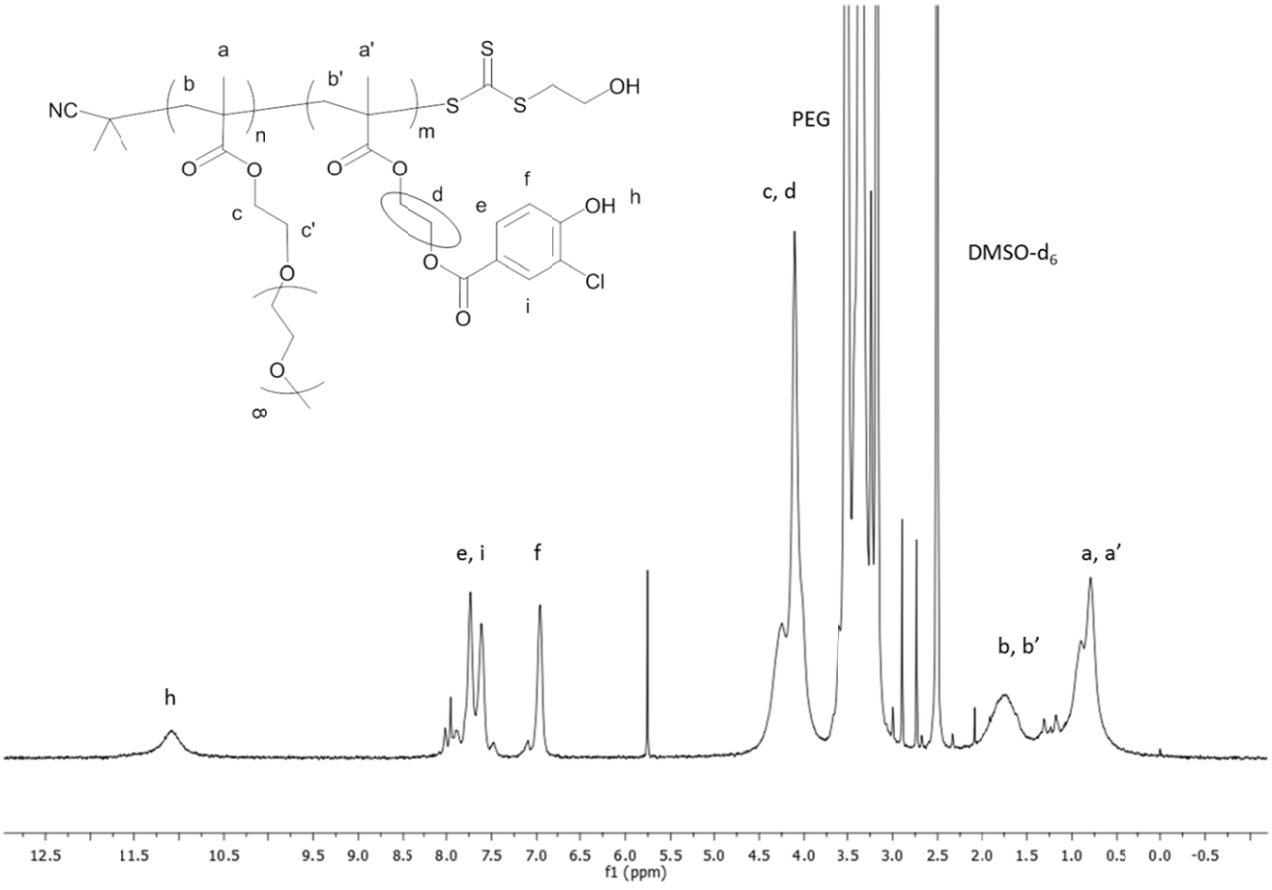


Figure S29. ^{1}H NMR spectrum of PEGMA$_{11}$-*b*-MCH$_{21}$ **P14** in DMSO-d$_6$.

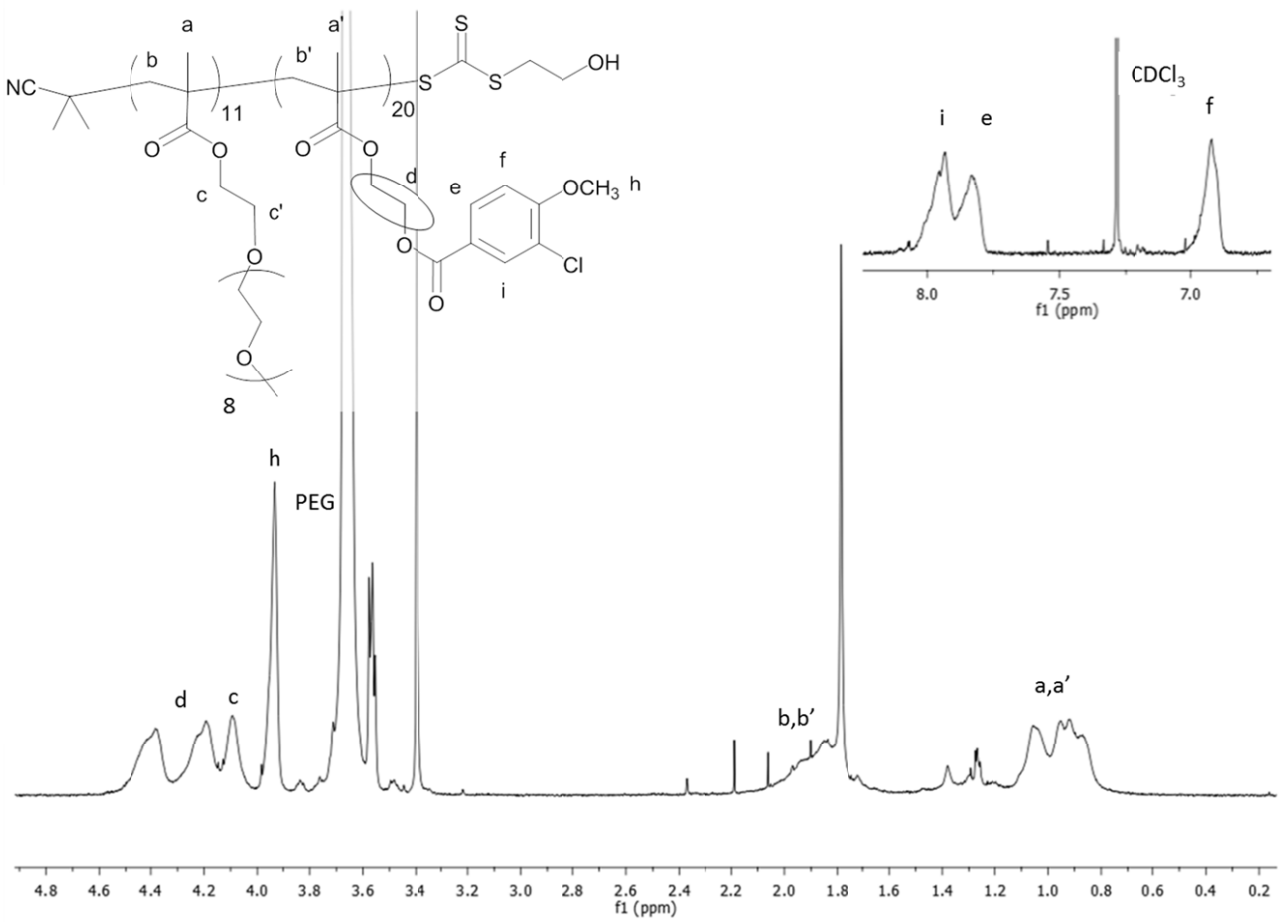


Figure S30. ^{1}H NMR spectrum of PEGMA$_{11}$-*b*-MCM$_{20}$ **P16** in DMSO-d$_6$.

5.0 Physical characterization of assembled nanostructures obtained from poly(GMA-*b*-MCH) and poly(PEGMA-*b*-MCH) copolymers.

5.1 Dynamic Light Scattering, turbidrimetric assays and potentiometric titration of random and block poly(GMA-*co*-MCH) copolymers.

20 mg mL^{-1} aqueous solution of random or block poly(GMA-*co*-MCH) copolymers at pH 12 (milliQ water + 1 N NaOH) were diluted to a concentration of 1.0 mg mL^{-1} with the following buffers: 0.05 M borate pH 9; 0.05 M phosphate pH 7.4, and 6.5; 0.05 M acetate pH 5 and 4. The solutions were analyzed by Dynamic Light Scattering at a 90° fixed angle with a Viscotek DLS

instrument. Particle size distributions were derived from correlation functions obtained using OmniSIZE 2.0 software.

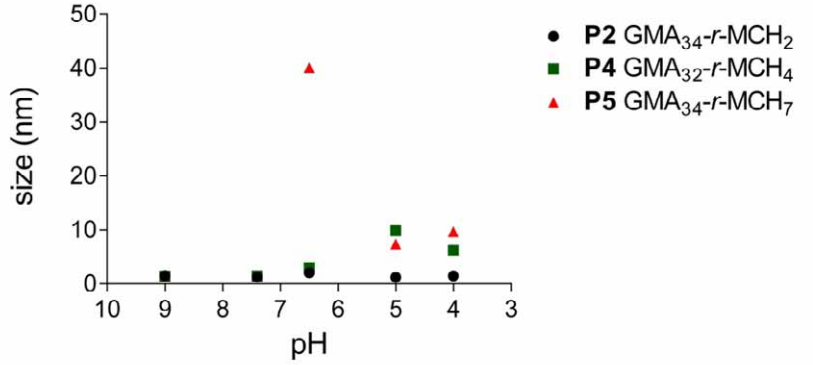


Figure S31. DLS size analysis reported in volume of solutions of poly(GMA-*r*-MCH) random copolymers in the 4-9 pH range. (●) GMA_{34}-*r*-MCH_2 **P2**, (■) GMA_{32}-*r*-MCH_4 **P4**, (▲) GMA_{34}-*r*-MCH_7 **P5**.

P2, **P4** and **P5** poly(GMA-*b*-MCH) random copolymer (Figure S31) were found to be in unimeric form, with a size of about 2 nm, in the 7.4-9.0 pH range. At pH 6.5, aggregation to give 40 nm assemblies was observed for copolymer **P5** (red spots on the graph). The correlation curve (data not showed) exhibited two exponential decays, one for faster moving aggregate of smaller size and one for slow-moving bigger particles. The formation of a precipitate was observed. Upon further decrease in pH, and consequent increase in hydrophobicity, the colloidally stable assemblies reached a size of about 10 nm, while an increase in the amount of precipitated macro-aggregates was observed. **P4** random copolymer, characterized by a lower content in MCH hydrophobic monomer compared to **P5**, showed aggregation below pH 6.5, in agreement with the turbidimetric measurements, where a CP at around pH 6.3 was detected. .

The same analysis was performed on poly(GMA-*b*-MCH) block copolymers (Figure S32). In this case the amphiphilic nature of the copolymers and ordered composition could induce the formation of colloidal system.

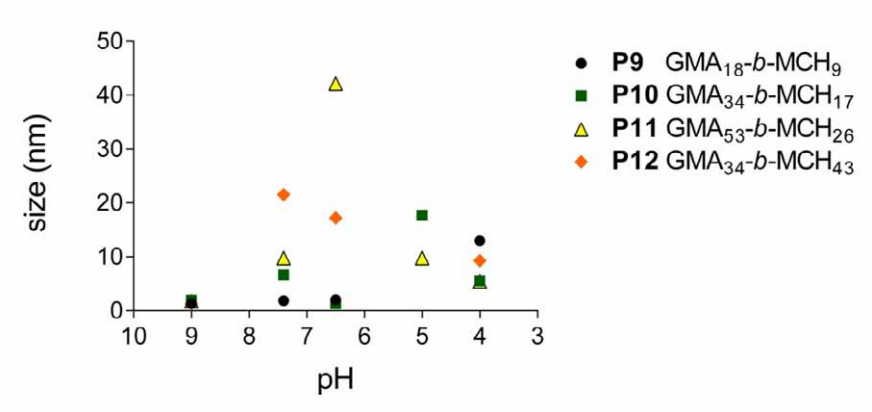


Figure S32. DLS size analysis reported in volume of solutions of poly(GMA-*b*-MCH) block copolymers in the 4-9 pH range. (●) GMA_{18}-*b*-MCH_9 **P9**, (■) GMA_{34}-*b*-MCH_{17} **P10**, (▲) GMA_{53}-*b*-MCH_{26} **P11**, (♦) GMA_{34}-*b*-MCH_{43} **P12**. At pH 5 **P12** formed macroaggregates of 10 μm, not reported in the graph.

Table S6 DLS analysis and CMC values for poly(GMA-*b*-MCH) block copolymers **P11** and **P12**.

Code	Polymers	Size (nm)[a]	PDI	CAC (μM)[a]
P11	GMA_{53}-*b*-MCH_{26}	55.4	0.314	5.7
P12	GMA_{34}-*b*-MCH_{43}	100.2	0.223	7.2

[a]DLS and CMC measurements were performed in PBS, pH 7.4, 25°C.

Polymers GMA_{53}-*b*-MCH_{26} **P11** and GMA_{34}-*b*-MCH_{43} **P12** were observed to strongly respond to pH changes. Their ability to self-assembly in buffer mimicking physiological conditions (PBS, pH 7.4) was investigated and their CAC (Figure S33) was calculated as described in the section "Fluorescence spectroscopy: Critical Aggregation Concentration".

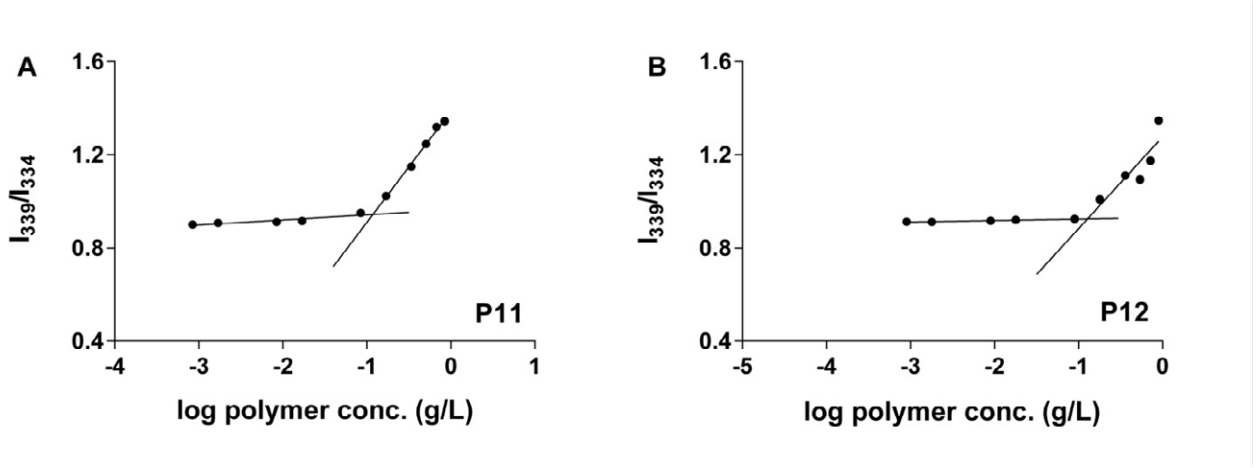


Figure S33. CAC profile of poly(GMA-*b*-MCH) block copolymers (A) GMA_{53}-*b*-MCH_{26} **P11** and (B) GMA_{34}-*b*-MCH_{43} **P12** obtained by pyrene method.

CAC values were found to be 5.7 and 7.2 μM for GMA_{53}-*b*-MCH_{26} **P11** and GMA_{34}-*b*-MCH_{43} **P12**, respectively. The low CAC values ensure good stability of the colloidal systems even in diluted conditions, that is prerequisite for the development of polymeric nanocarriers. DLS analysis were carried out on samples prepared for the CAC measurement (Figure S34) at a 50μM polymer concentration, in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.4.

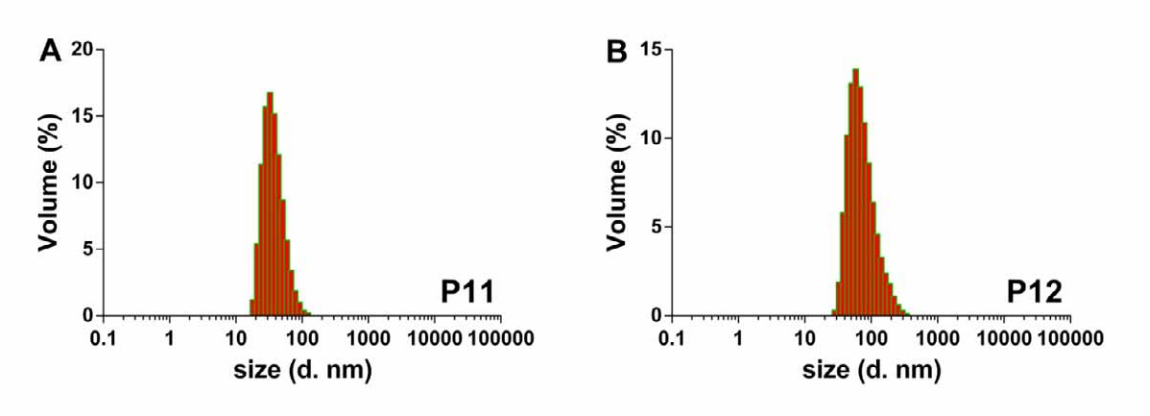


Figure S34: DLS analysis reported in volume of colloidal assemblies formed by poly(GMA-*b*-MCH) block copolymers GMA_{53}-*b*-MCH_{26} **P11** and GMA_{34}-*b*-MCH_{43} **P12**.

The sizes detected were different from the data obtained with our previous measurements. **P11** and **P12** size increased from 10 to 50 nm and from 22 to 100 nm, respectively, which could be ascribed to the non-negligible change in the ionic strength used for the analysis (50 mM in the first set of measurements, 150 mM for CAC experiments).

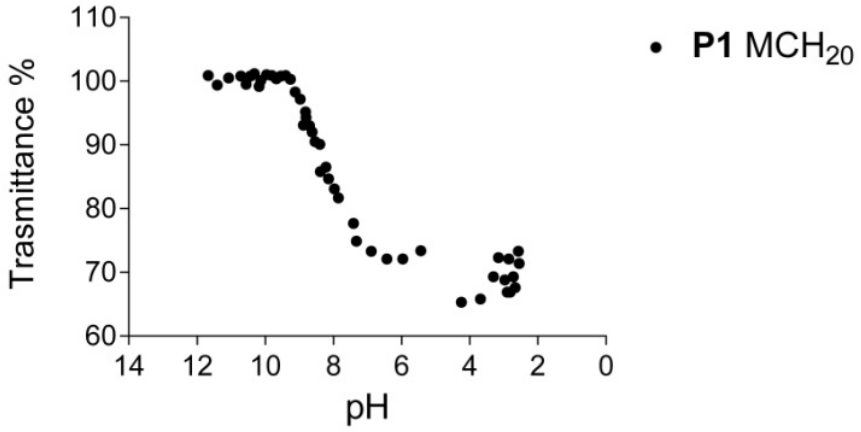


Figure S35. Turbidimetric profile of pMCH homopolymer **P1** at polymer concentration of 1.0 mg mL^{-1} in deionized water.

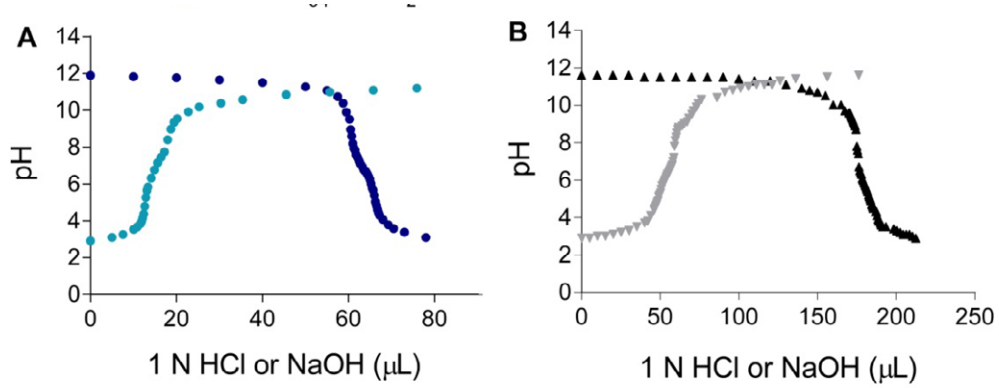


Figure S36. Titration and backtitration curves for (A) GMA_{34}-*r*-MCH_2 **P2**, and (B) GMA_{35}-*r*-$MDCH_2$ **P3** random copolymers.

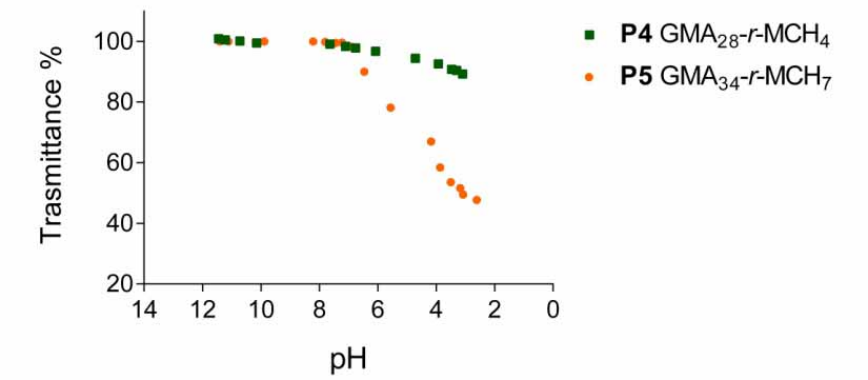


Figure S37. Turbidimetric profile of (■)GMA_{32}-*r*-MCH_4 **P4** and (●)GMA_{34}-*r*-MCH_7 P5 random copolymers in the 3-11 pH range.

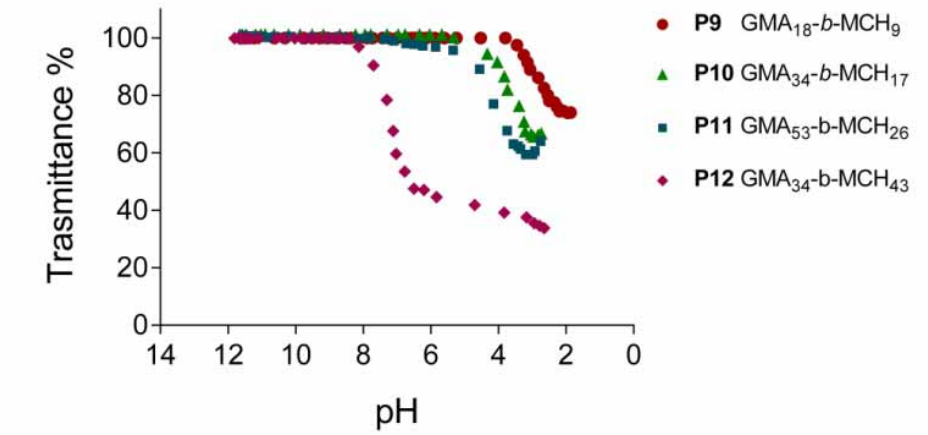


Figure S38. Turbidimetric analysis of poly(GMA-*b*-MCH) block copolymers. 1.0 mg mL^{-1} polymer solutions in milliQ water at pH 12.0 were gradually acidified with 1.0 N HCl until pH 3.0. Cloud points (CP) indicates the pH at which a decrease in transmittance (λ = 500 nm) starts to be detected.

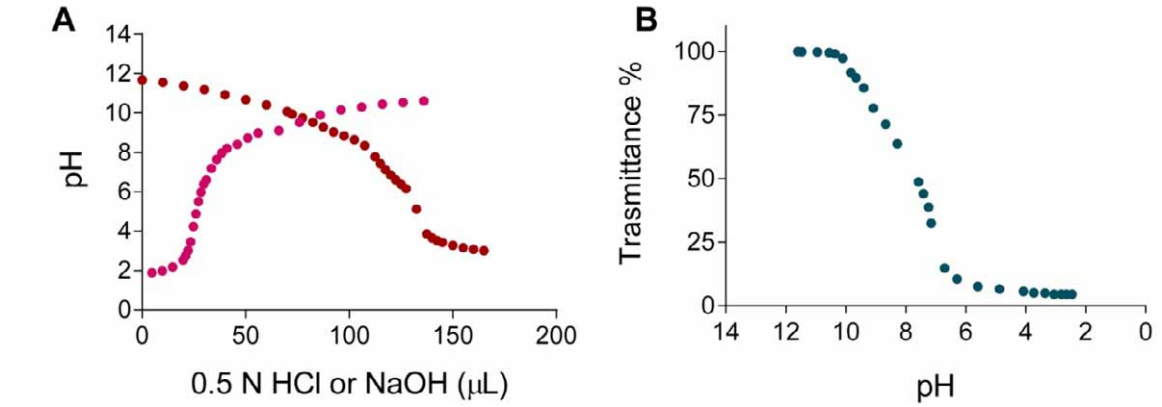


Figure S39. (A) Potentiometric titration profile of $PEGMA_{11}$-*b*-MCH_{21} **P14** diblock copolymer in deionized water at 1.0 mg mL^{-1} polymer concentration. The average value from duplicates was plotted; (B) Turbidimetric profile of $PEGMA_{11}$-*b*-MCH_{38} **P15** at 1.0 mg mL^{-1} polymer concentration, in the 2-12 pH range. Trasmittance % was measured at λ=500 nm.

6. References

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