Supporting Information

Size-Controlled Clustering of Iron Oxide Nanoparticles within Fluorescent Nanogels using LCST-Driven Self-Assembly

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EXPERIMENTAL SECTION

Materials

The following reagents were used as received: Carbon disulfide (Sigma-Aldrich, 98%); iron chloride ($FeCl_3 \cdot 6H_2O$, Sigma-Aldrich, 97%); sodium oleate (Sigma-Aldrich, 97%); iodine (Sigma-Aldrich, 99%); 2,3-dichloromaleic anhydride (Sigma Aldrich, 97%); 3-amino-1-propanol (Sigma Aldrich, 99%); 4,4′-azobis(4-cyanovaleric acid) (ACVA, Sigma Aldrich, 98%); methacryloyl chloride (Sigma Aldrich, 97%); dopamine hydrochloride (Sigma Aldrich); 1-octadecene (VWR International Ltd.); N-hydroxysuccinimide (NHS, Sigma Aldrich, 98), *N,N′*-dicyclohexylcarbodiimide (DCC, Sigma Aldrich, 99%); triethylamine (Sigma Aldrich, 99%);

2,2'-(ethylenedioxy)bis(ethylamine) (Sigma Aldrich, 98%); poly(ethylene glycol) monomethyl ether methacrylate (OEGMA, M_n = 300 g mol^{-1}, Sigma Aldrich); 1,3,5-trioxane (Sigma Aldrich, 99%). 2,2' azobisisobutyronitrile (AIBN) was received from Sigma-Aldrich (98%), recrystallized from methanol and stored in the dark at 4 °C. Solvents were purchased from Fisher Scientific and used as received. Monomers and 1,4-dioxane were filtered through a plug of basic alumina prior to use and stored at 4 °C. Deuterated solvents and silica gel (40-63 μm) were used as received from Apollo Scientific.

Instruments and Methods

^{1}H-NMR and ^{13}C-NMR spectra were recorded at 300 MHz or 400 MHz on a Bruker DPX-300 or a Bruker DPX-400 spectrometer, using chloroform-*d* ($CDCl_3$) as solvent.

Size exclusion chromatography (SEC) analysis was performed on a system composed of an Agilent 1260 Infinity II LC system equipped with an Agilent guard column (PLGel 5 μM, 50 × 7.5 mm) and two Agilent Mixed-C columns (PLGel 5 μM, 300 × 7.5 mm). The mobile phase used was THF (HPLC grade) containing 2% v/v NEt_3 at 40 °C at flow rate of 1.0 mL min^{-1} (polystyrene (PS) standards were used for calibration).

Turbidimetry analysis of polymer was performed on an Evolution 350 UV-Vis spectrophotometer fitted with a heating and cooling system. Polymer sample was prepared at 2 mg mL^{-1} with the transmittance of the sample measured at a wavelength of 500 nm over two heating and cooling cycles at a heating and cooling rate of 1 °C min^{-1}. The cloud point temperature (T_{CP}) was defined as the temperature where the transmittance decreased to 50% in the second heating run.

Dynamic light scattering (DLS) was performed on a Malvern Zetasizer Nano ZS with a 4 mW He-Ne 633 nm laser module operating at 25 °C. After an equilibration time of 30 s, at least 10 runs were carried out at 25 °C. The measurements were performed at an angle of 173°. Each measurement was done in triplicate. The size distribution of suspensions were calculated applying the nonlinear least-squares fitting mode. The Z-average diameter and the width of the

distribution as the polydispersity index of the particles (PD) were obtained by the cumulants method assuming a spherical shape of the particles.

Electrophoretic light scattering was used to measure the electrokinetic potential, also known as ζ-potential. The measurements were performed on a Malvern Zetasizer Nano ZS instrument by applying laser Doppler velocimetry. For each measurement, at least 30 runs were carried out using at 150 V. Each experiment was performed in triplicate at 25 °C. Zeta potential was calculated from the corresponding electrophoretic mobilities (μE) by using the Henry's correction of the Smoluchowski equation ($\mu E = 4\pi\ \varepsilon 0\ \varepsilon r\ \zeta\ (1+\kappa r)/6\pi\ \mu$).

UV-Vis spectroscopy was performed on Evolution 350 UV-Vis spectrophotometer for examining the absorption spectral data using Thermo INSIGHT-2 v.10.0.30319.1 software. Fluorescence spectral data was determined using an Agilent Cary Eclipse Fluorescence spectrophotometer equipped with Photomultiplier tube (PMT) detector. Quartz cuvettes from Starna scientific (Type: 3/Q/10) with four polished sides were used for fluorescence.

All steady state emission and excitation were obtained with an Edinburgh Instruments FS5 Spectrofluorometer and analysed in Fluoracle (Edinburgh Instruments) and Origin 2019 (Origin Labs) to examine the photophysical properties of fluorescent nanogels.

Thermogravimetric analysis (TGA) was performed on a TG550 (TG instruments) in the temperature range of 20 to 600 °C with a heating rate of 20 C min^{-1}. The weight loss percentage was calculated by the difference between the sample weights at 20 °C and at 570 °C

Samples for TEM analysis were prepared by drop casting 7 μL of sample (0.5 mg/mL) onto a carbon/formvar-coated copper grid placed on filter paper. Samples were stained with a 1% uranyl acetate solution to facilitate imaging of the thin organic structures unless specified. Imaging was performed on a Jeol 2100 transmission electron microscope operating at 120 kV.

Synthesis

Synthesis of dichloromaleimide functional monomer. The dichloromaleimide functional monomer was synthesized in a two-step reaction as demonstrated in Scheme S1. First, 2,3-dichloromaleic anhydride (**1**) (3.0 g, 17.96 mmol) and 3-aminopropan-1-ol (1.48 g, 19.76 mmol)

in acetic acid (20 ml) was heated at 80 °C for 3.5 h. Acetic acid was evaporated using a rotary evaporator and the product, 3,4-dichloro-1-(3-hydroxypropyl)-1H-pyrrole-2,5-dione, (**2**) was then purified by silica gel column chromatography using hexane (Hxn) and ethylacetate (EA) at varying volume ratios (Hxn : EA = 3 : 1; 2 : 1; 1 : 1) (Yield: 39%). The product (**2**) was characterized using ^{1}H and ^{13}C spectroscopy (Figure S1 and S2, accordingly). ^{1}H NMR (300 MHz, $CDCl_3$) 3.76 (2 H, q, J 6.5), 3.68 – 3.58 (2 H, m), 1.89 (1 H, s), 1.84 (2 H, ddd, J 9.0, 7.6, 4.3).

Then, 3,4-dichloro-1-(3-hydroxypropyl)-1H-pyrrole-2,5-dione (**2**) (3.0 g, 13.39 mmol) and triethylamine (1.76 g, 17.40 mmol) were dissolved in dichloromethane (30 mL) at 0 °C. Finally, methacryloyl chloride (1.82 g, 17.40 mmol) was added dropwise into the solution. The final solution was stirred for 30 min at 0 °C for a further 6 h at room temperature. After the reaction, the solution was filtered to remove the salt formed during the reaction. The solvent was evaporated using a rotary evaporator. The crude product was then purified by silica gel column chromatography to give the product as a yellow solid (Hxn : EA = 8 : 1) (Yield: 68%). ^{1}H and ^{13}C NMR spectroscopy analysis proved the successful synthesis of the desired monomer (Figure S3 and S4, accordingly). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 6.10 – 6.05 (1 H, m), 5.62 – 5.52 (1 H, m), 4.25 – 4.07 (2 H, m), 3.74 (2 H, t, J 6.9), 2.03 (2 H, ddd, J 9.5, 8.1, 4.6), 1.95 – 1.91 (3 H, m).

Synthesis of dopamine functional CTA. The dopamine functional CTA was synthesized as demonstrated in Scheme S1. First, a carboxylic acid functional CTA, 4-cyano-4-(((ethylthio)carbonothioyl)thio)pentanoic acid, (**3**) was synthesized as reported before.[1] The CTA was characterized using ^{1}H and ^{13}C spectroscopy (Figure S5 and S6, accordingly). Then, based on a literature method, the carboxylic acid functional CTA was further reacted with N-

hydroxysuccinimide (NHS) to obtain NHS functional CTA.[2] The synthesized NHS functional CTA was characterized using ^{1}H and ^{13}C spectroscopy (Figure S7 and S8, accordingly). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.43 – 3.27 (2 H, m), 2.97 – 2.89 (2 H, m), 2.85 (4 H, s), 2.72 – 2.46 (2 H, m), 1.88 (3 H, s), 1.39 – 1.33 (3 H, m).

Finally, dopamine hydrochloride (0.53 g, 2.77 mmol), NHS functional CTA (0.80 g, 2.22 mmol) and triethylamine (0.3 g, 3.02 mmol) were dissolved in 30 mL methanol and stirred for 48 h at ambient temperature. After 48 h, the solvent was removed under reduced pressure and the product, 2-cyano-5-((3,4-dihydroxyphenethyl)amino)-5-oxopentan-2-yl ethyl carbonotrithioate, was then purified by silica gel column chromatography (Hxn : EA = 3 : 1) (Yield: 68%). Characterization by ^{1}H and ^{13}C NMR spectroscopy confirmed the desired structure (Figure S9 and S10, accordingly). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 6.80 (1 H, d, J 8.0), 6.71 (1 H, d, J 2.0), 6.57 (1 H, dd, J 8.0, 2.0), 5.90 (1 H, t, J 5.7), 4.20 – 4.06 (1 H, m), 3.46 (2 H, q, J 6.6), 3.33 (2 H, q, J 7.4), 2.69 (2 H, t, J 6.9), 2.57 – 2.22 (4 H, m), 1.89 – 1.78 (3 H, m), 1.34 (3 H, dd, J 9.5, 5.4).

Synthesis of IONPs. Iron oxide nanoparticles were synthesized using a modified literature method.[3] The synthesis of iron oxide nanoparticles contains two steps. Briefly, 2.00 g (7.4 mmol; 1.0 eq.) of iron chloride ($FeCl_3 \cdot 6H_2O$) was dissolved in in a mixture solvent composed of 15 ml ethanol, 10 ml distilled water and 25 ml hexane in a 250 mL of roundbottom flask, and mixed with 6.76 g (22.2 mmol; 3.0 eq.) of sodium oleate. The obtained solution was heated to 70 °C and kept at that temperature for 4.5 hours. Subsequently, the organic phase was separated after washing with 20 ml of water three times. Iron oleate was obtained in a waxy by solid form after removing hexane under reduced pressure and used for the next step (6.0 g; 6.66 mmol; yield 90%). 2.0 g of (2.22 mmol) the obtained iron oleate complex mixed with oleic acid (0.344 g, 1.22 mmol) was added to a three-necked flask (25 mL) with a solvent of 1-octadecene (10 g) at

room temperature. The reaction mixture was heated to 150 °C at a heating rate of ~10 °C/min and kept at that temperature for 10-15 min. Afterwards, the reaction mixture was heated to 320 °C and was aged at that temperature for further 30 min. The resulting solution was quickly cooled to room temperature followed by the purification with acetone/hexane (precipitation/redispersion) for 3-5 rounds and dispersed in THF for further use.

Synthesis of statistical copolymers. RAFT copolymerization procedure (Table S1, **P1**) is as follows: 960 mg $OEGMA_{300}$ (3.2 mmol), 234 mg DCMMA (0.8 mmol), 15.94 mg CTA (0.04 mmol) and 1.64 mg AIBN (0.01 mmol) were dissolved in dioxane in a schlenk flask equipped with a magnetic stir bar. The total volume of the reaction mixture was 10 mL. The reaction was degassed via three freeze-pump-thaw cycles and then placed under nitrogen atmosphere, the t_0 sample for ^{1}H NMR was taken, and the flask was immersed in a pre-heated oil bath under stirring at 70 °C. After 15.5 h, the polymerization was stopped by cooling to room temperature and exposing to air. The monomer conversions for $OEGMA_{300}$ (M1) and DCMMA (M2) were determined as 62% and 68%, respectively, by ^{1}H NMR using 1,3,5-trioxane as an internal standard. The theoretical molar mass ($M_{n,theo.}$) of the copolymer was calculated as 19,250 g mol^{-1} by the formula $M_{n,theo.} = [([M]_{M1}/[CTA] \times Conv. \times M_{M1}) + ([M]_{M2}/[CTA] \times Conv. \times M_{M2}) + (M_{CTA})]$ (Formula 1). The polymer was purified by precipitating two times in hexane:diethyl ether (3:1). The resulting yellow colored polymer was dried under high vacuum at room temperature until constant weight. The number-average molar mass (M_n) and molar mass dispersity ($Đ_M$) were determined by SEC using PS standards.The degree of polymerization (DP) for each polymer was calculated from the signal integrals of the ^{1}H NMR spectrum (Figure S11) of the purified copolymer using the following equations:

$$DP_{OEGMA300} = \frac{I(signal\ 2)/\ 2}{I(signal\ 1)/\ 3}$$ (Formula 2)

$$DP_{DCMMA} = \frac{I(signal\ 3)/2}{I(signal\ 1)/\ 3}$$ (Formula 3)

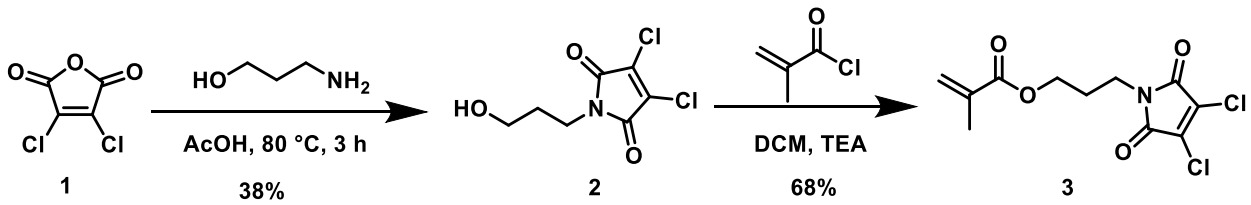
with *I(signal 2)* corresponding to the integral of the methine proton peaks of the $OEGMA_{300}$ at 4.0 ppm, *I(signal 3)* corresponding to the integral of methyl proton peaks of the DCMMA at 3.90 ppm, and *I(signal 1)* corresponding to the integral of α-RAFT end groups (δ = 6.73, 6.65, and 6.51 ppm). Number average molar mass values were calculated by using the following equation: $M_{n,NMR} = (DP_{OEGMA300} \times M_{OEGMA300}) + (DP_{DCMMA} \times M_{DCMMA}) + M_{CTA}$ (Formula 4).

Surface modification of IONPs. Briefly, IONPs (42.5 mg) and the synthesized polymer (125 mg) were dispersed in THF (20 mL), and the mixture was incubated for 48 h. The solvent was evaporated and the IONPs were washed with THF for 5 times to remove excess polymers.

Synthesis of polymer nanogels. A typical procedure for nanogel synthesis is as follows: in a glass vial, polymer (2 mg) was dissolved in water (1 mL) and heated at 70 °C for 1.5 min. After 1.5 min, 10 μL of 2,2′-(ethylenedioxy)bis(ethylamine) aqueous solution (c = 20.9 mg mL^{-1}) was added into the polymer solution and the reaction was stirred at 70 °C for 20 min. The molar ratio of the dichloromaleimide and the crosslinker was set to 1:1. In order to check the reproducibility, this procedure was repeated 3 times. The obtained nanogels were characterized using DLS without any filtration.

Clustering of the polymer tethered IONPs. A typical procedure for hybrid-nanogel synthesis is as follows: Polymer tethered IONPs (**P-IONPs**) was dissolved in water (c = 2 mg mL^{-1}) in a glass vial and placed in a pre-heated oil bath at 70 °C for 1 min. After 1 min, 20 μL of 2,2′-

(ethylenedioxy)bis(ethylamine) aqueous solution (c = 20.9 mg mL^{-1}) was added into the solution and the reaction was kept at 70 °C for 5 minutes.



Scheme S1. Schematic representation of the synthesis of dichloromaleimide functional monomer DCMMA (**3**).

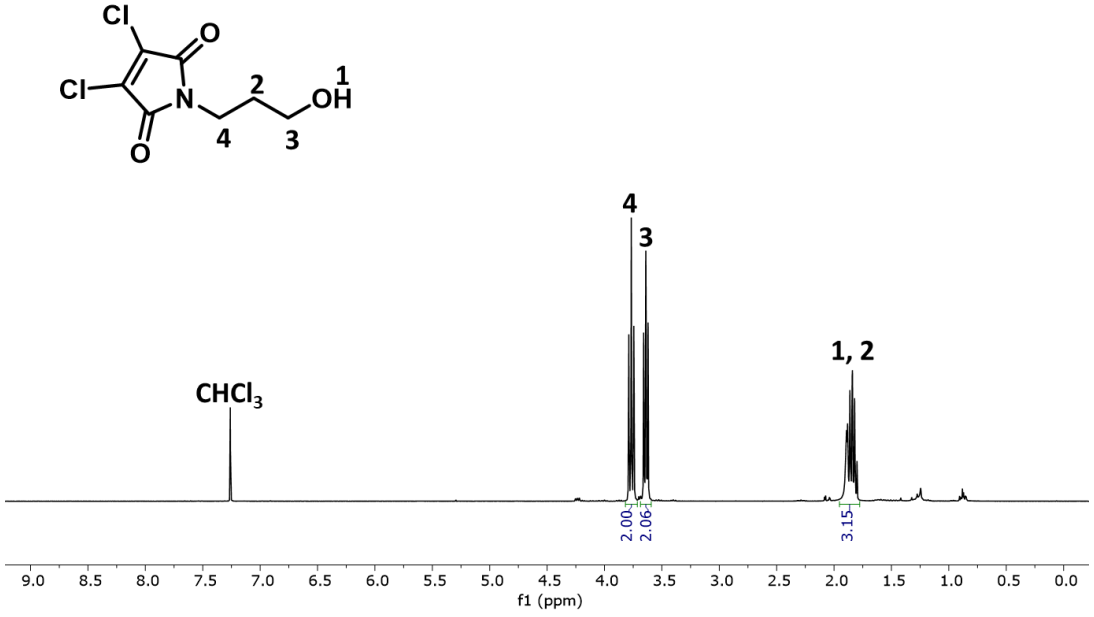


Figure S1. ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of 4-dichloro-1-(3-hydroxypropyl)-1H-pyrrole-2,5-dione.

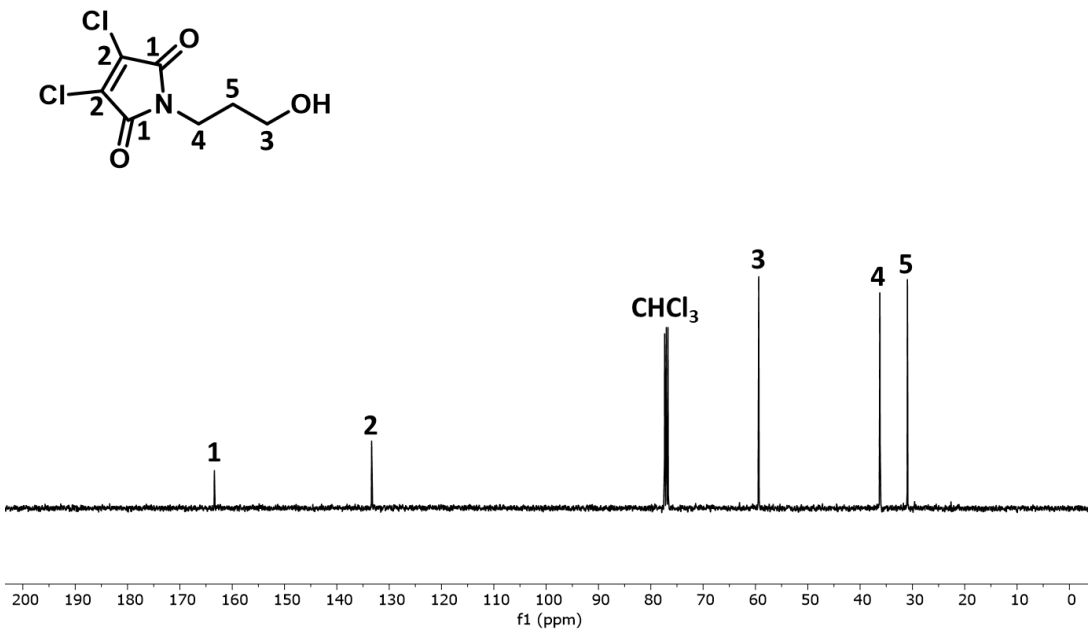


Figure S2. ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of 4-dichloro-1-(3-hydroxypropyl)-1H-pyrrole-2,5-dione.

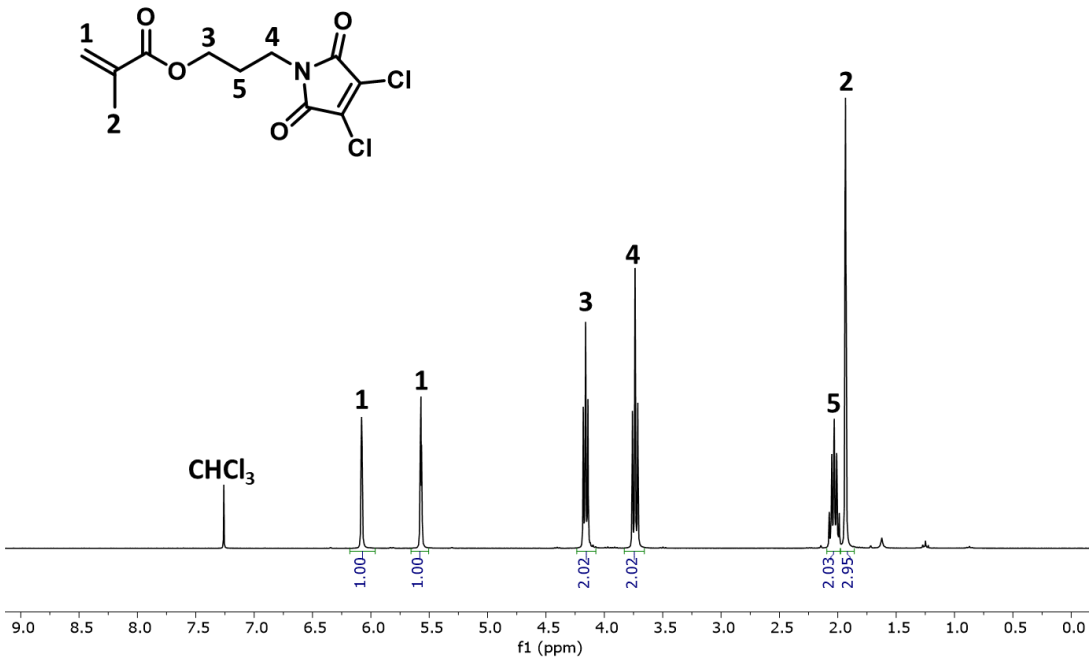


Figure S3. ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of the dichloromaleimide functional monomer DCMMA (**3**).

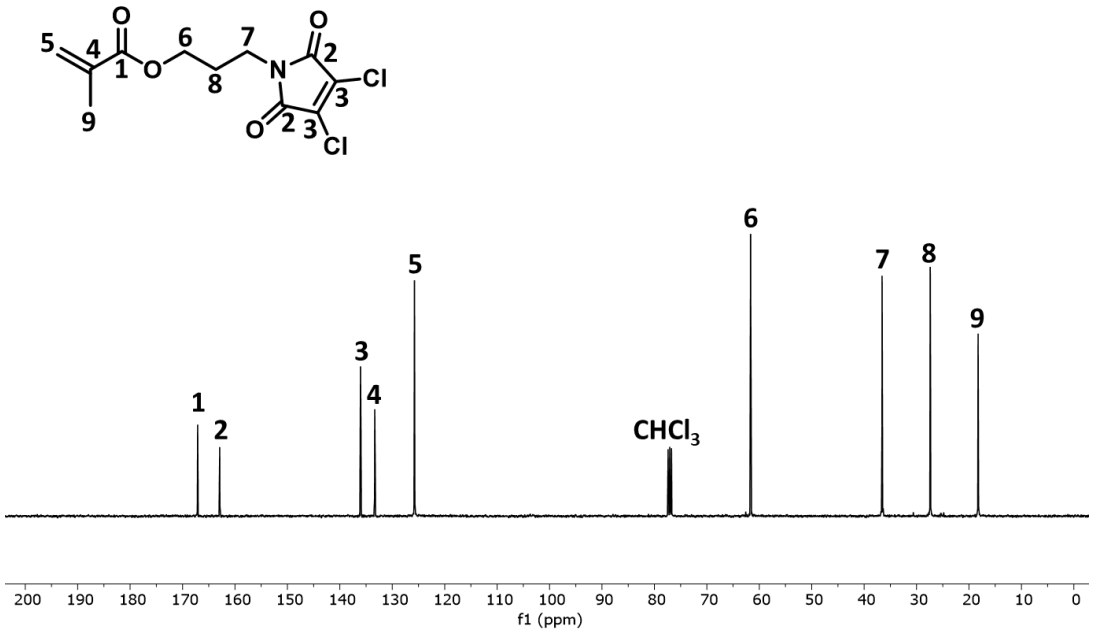
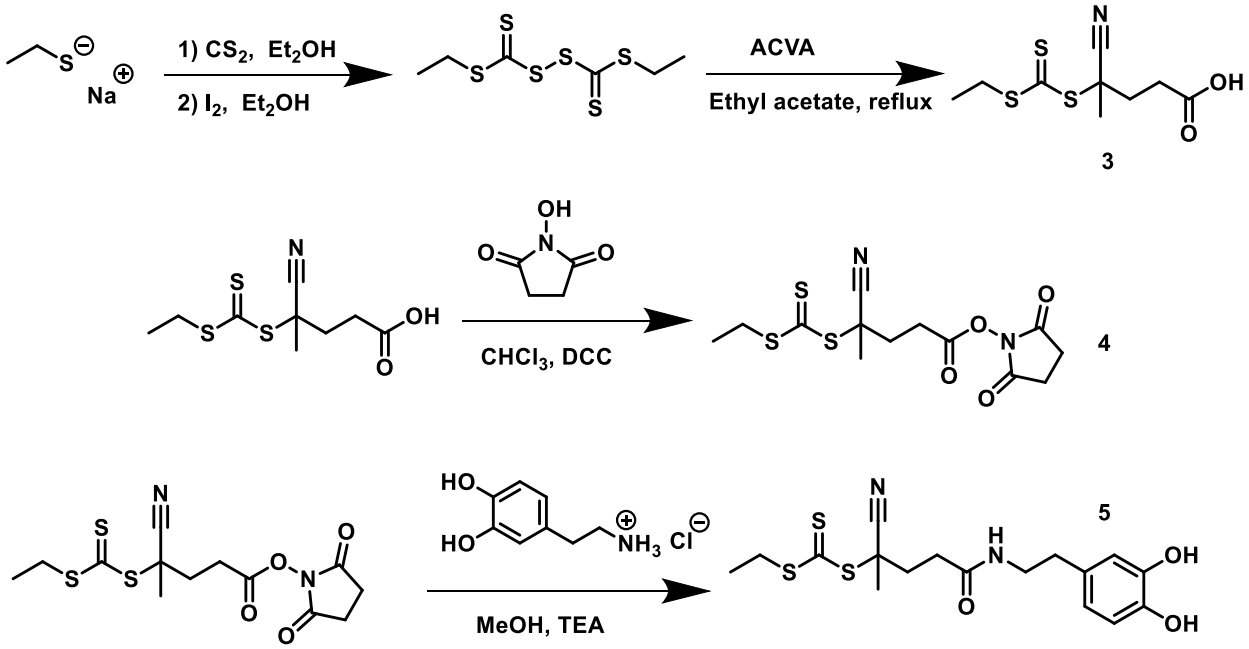


Figure S4. ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of the dichloromaleimide functional monomer DCMMA (**3**).



Scheme S2. Schematic representation of the synthesis of dopamine functional chain transfer agent (CTA).

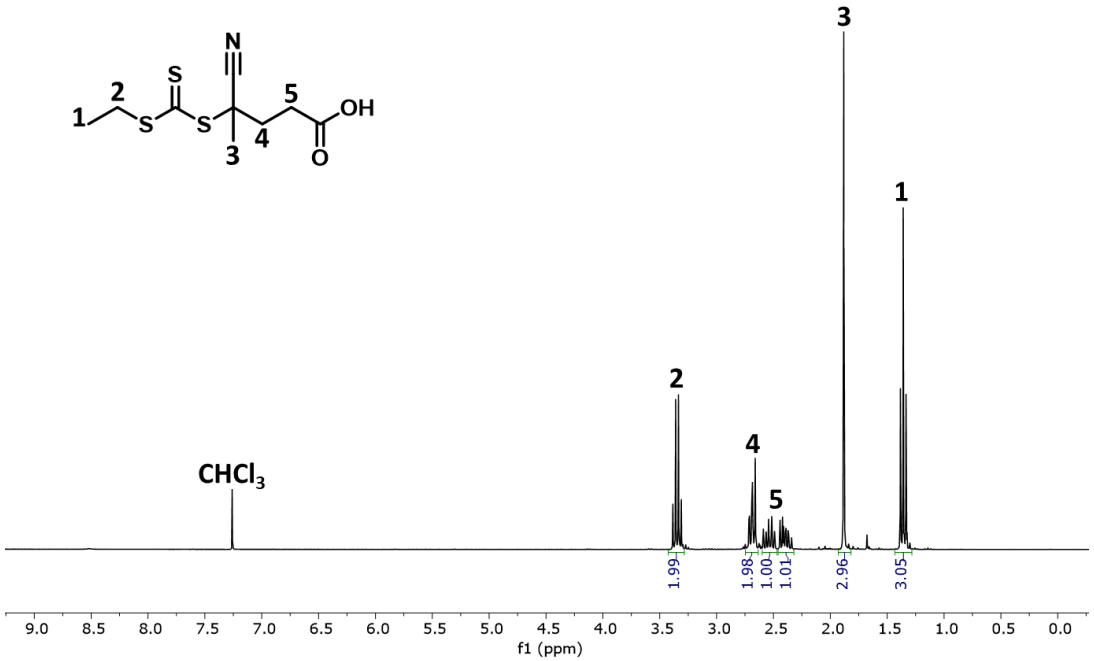


Figure S5. ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of the carboxylic acid functional CTA.

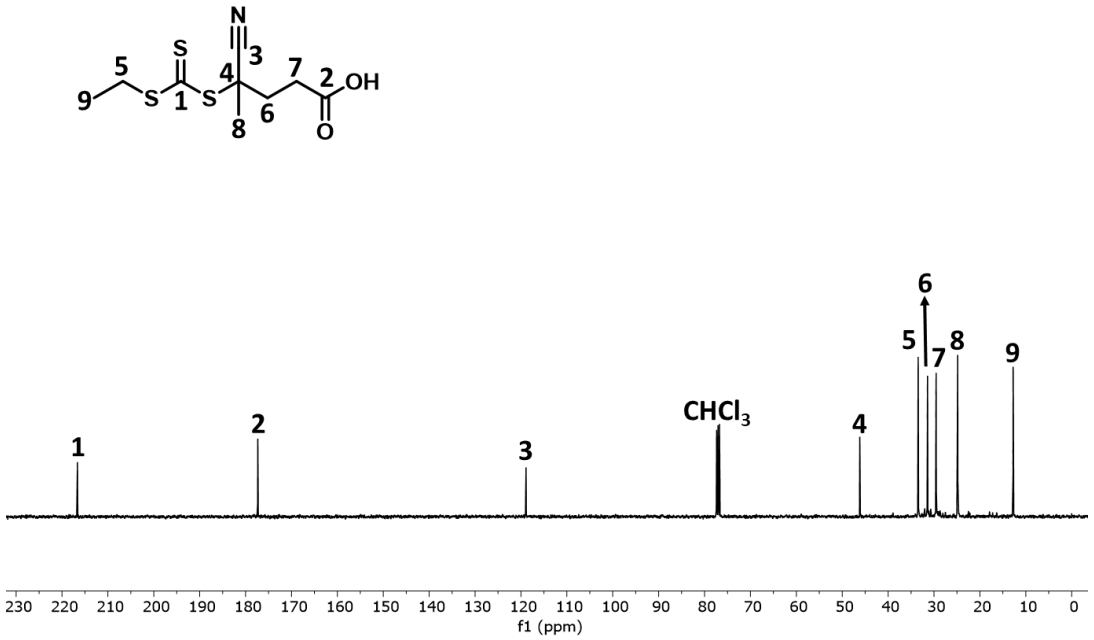


Figure S6. ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of the carboxylic acid functional CTA.

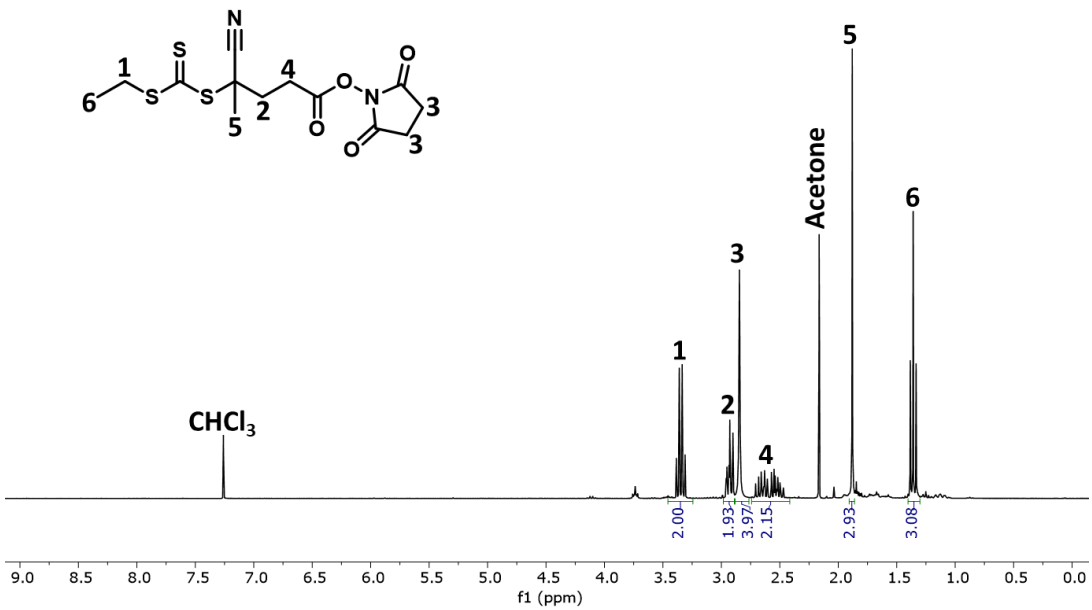


Figure S7. ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of the NHS functional CTA.

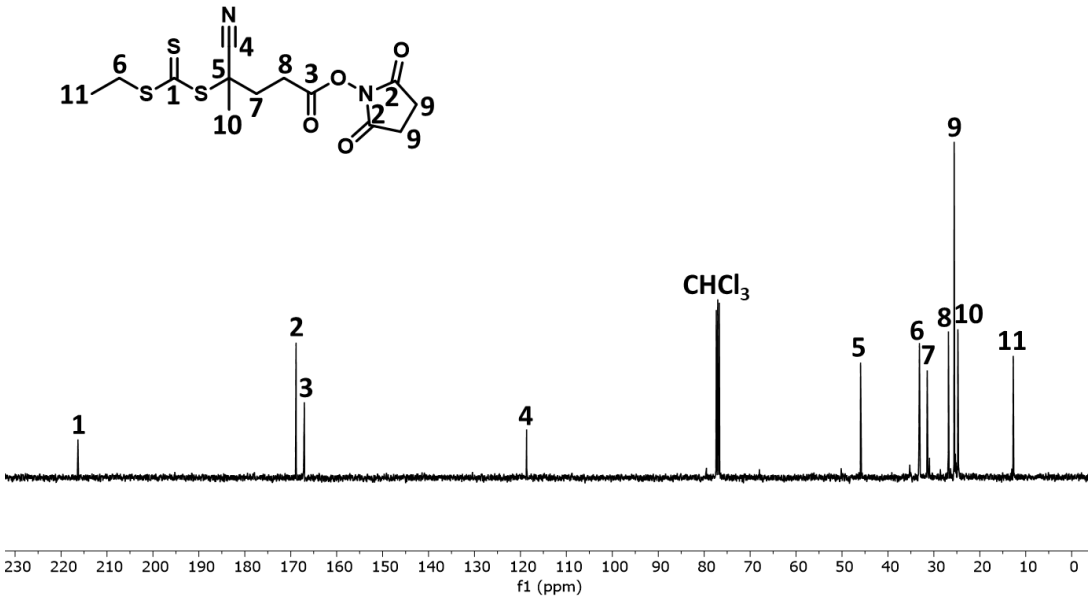


Figure S8. ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of the NHS functional CTA.

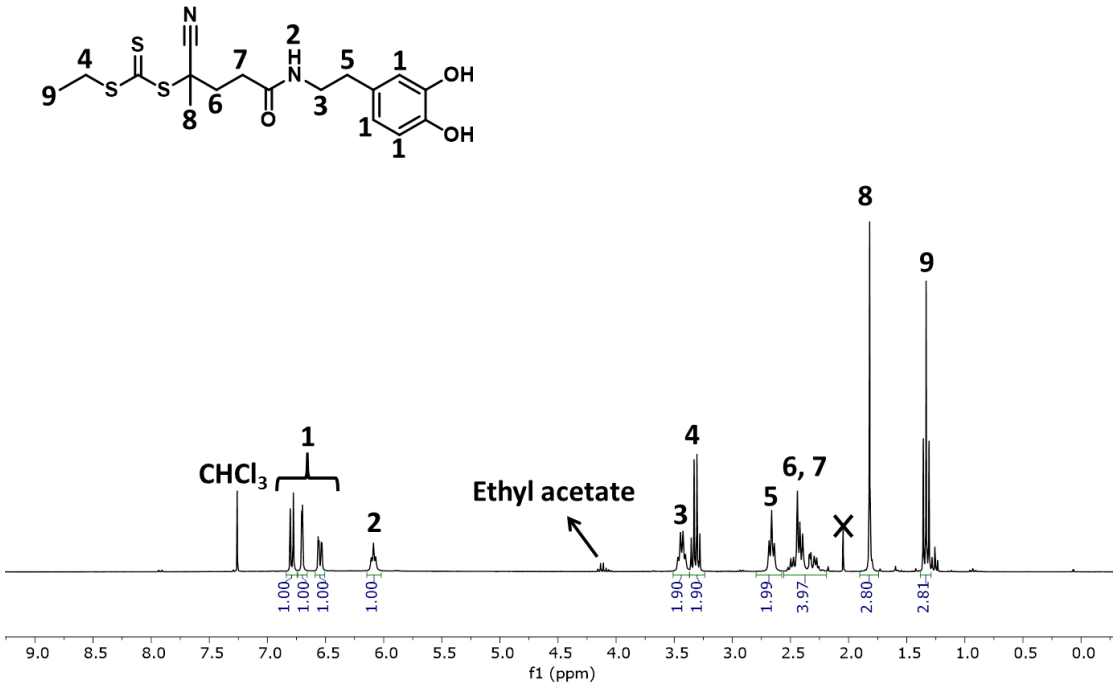


Figure S9. ^{1}H NMR spectrum (300 MHz, $CDCl_3$) of the dopamine functional CTA (**X** represents the residual acetone peak).

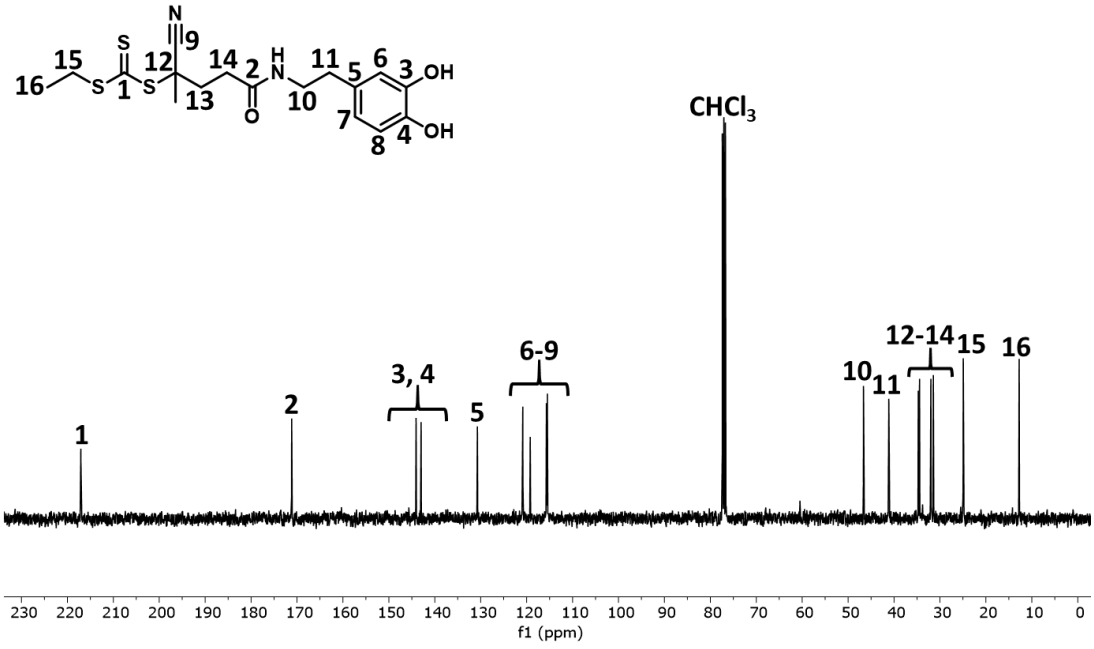


Figure S10. ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of the dopamine functional CTA.

Table S1. Selected characterization data for the copolymer of $OEGMA_{300}$ (M1) and DCMMA (M2).

Entry	M1/M2/ CTA[a]	CTA/ AIBN[b]	Pol. time (h)	Conv. [%][c] M1/M2	$M_{n,theo.}$[d] [g mol^{-1}][d]	DP[e] M1/M2	F(M1/M2)[f] [g mol^{-1}]	$M_{n,NMR}$[g] [g mol^{-1}]	$M_{n,SEC}$[i] [g mol^{-1}]	$Đ_M$[i]
P1	80/20/1	4	15.5	62/68	19,250	52/14	3.71	20,100	13,100	1.35

[a]Monomer to CTA molar ratio. [b]CTA to AIBN molar ratio. [c]Determined by ^{1}H NMR spectroscopy. [d]$M_{n,theo.}$ = [([M]$_{M1}$/[CTA] × Conv. × M_{M1}) + ([M]$_{M2}$/[CTA] × Conv. × M_{M2}) + (M_{CTA})]. [e]Determined from ^{1}H NMR spectrum of the isolated polymer. [f]Monomer ratio in the isolated copolymer. [g]$M_{n,NMR}$ = ($DP_{OEGMA300}$ × $M_{OEGMA300}$) + (DP_{DCMMA} × M_{DCMMA}) + M_{CTA}. [i]Determined by SEC in THF analysis (RI detection, PS calibration).

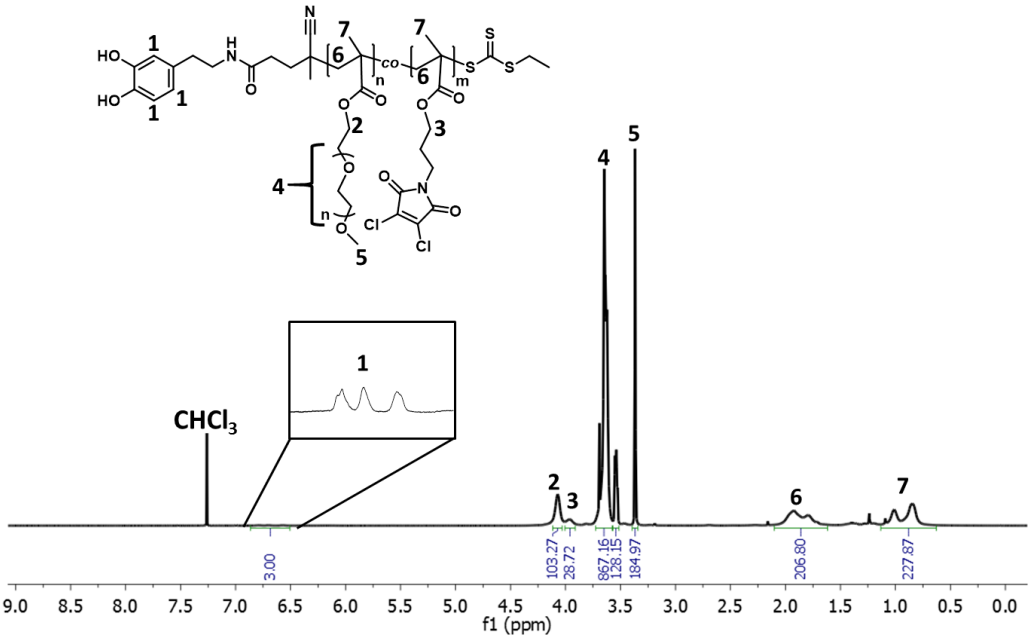


Figure S11. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of the isolated polymer.

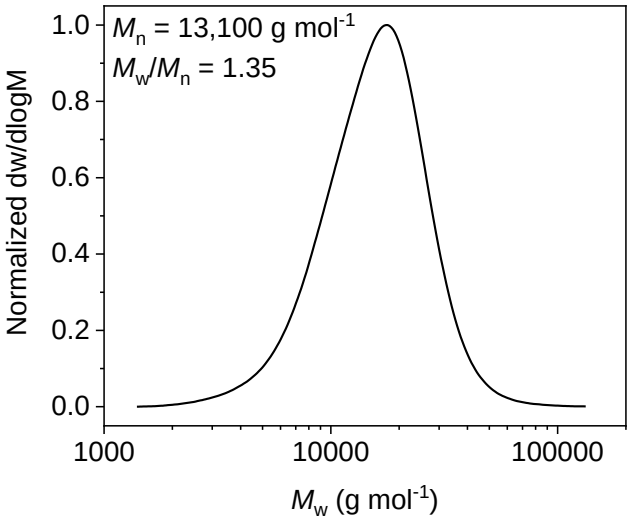
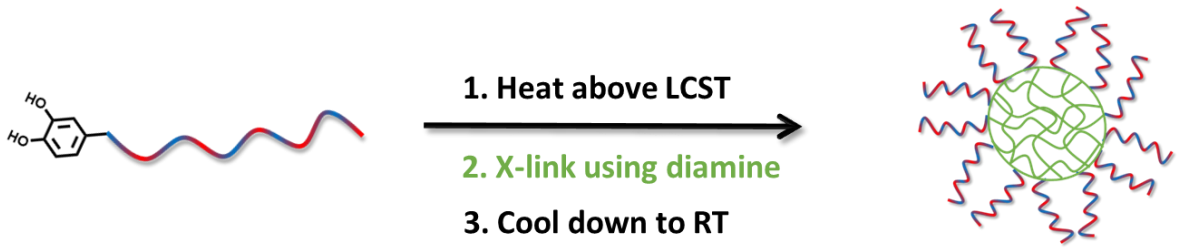


Figure S12. Normalized SEC trace of the isolated polymer in THF (PS calibration).



Scheme S3. Schematic representation of the nanogel synthesis.

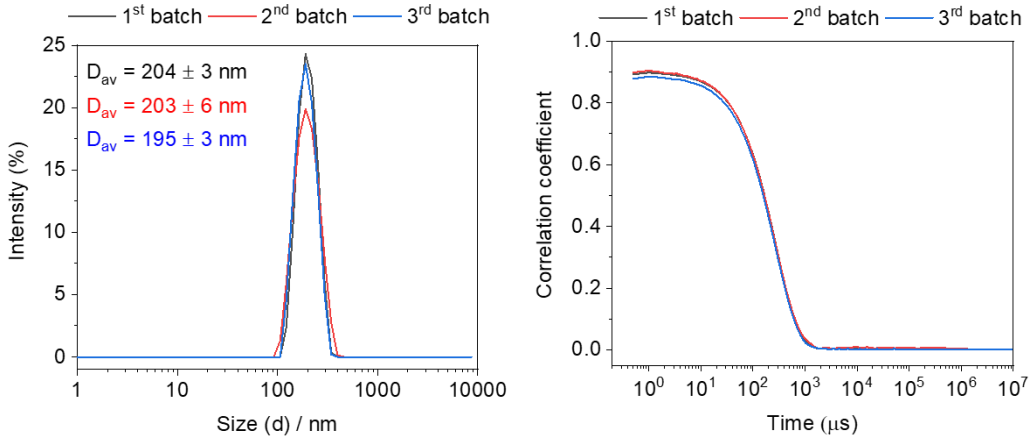


Figure S13. (Left) DLS size distributions (right) correlation function of nanogels synthesized with an initial polymer concentration of 2 mg mL^{-1}.

Table S2. Z-average size and polydispersity index (PD) values of the prepared nanogels.

Entry	Z-Average [d, nm]	PD
1st batch	194 ± 3	0.038 ± 0.010
2nd batch	188 ± 2	0.072 ± 0.019
3rd batch	187 ± 4	0.021 ± 0.015
After dialysis	193 ± 1	0.033 ± 0.024

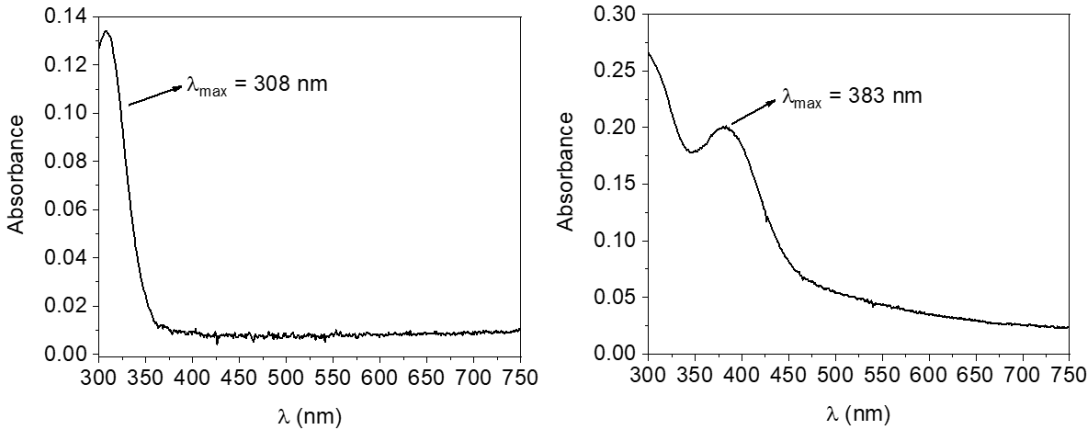


Figure S14. (Left) UV-vis spectrum of **P1** in water (c = 0.33 mg mL^{-1}). (Right) UV-vis spectrum of nanogels in water (c = 0.33 mg mL^{-1}).

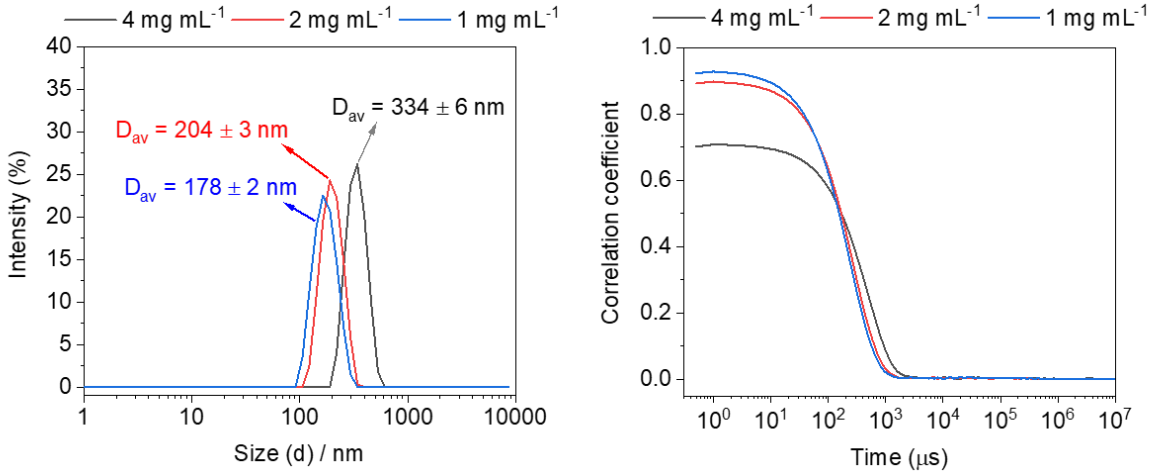


Figure S15. (Left) Intensity size distribution overlay and (right) correlation function overlay of nanogels in water with an initial **P1** concentration of 1, 2 and 4 mg mL^{-1}.

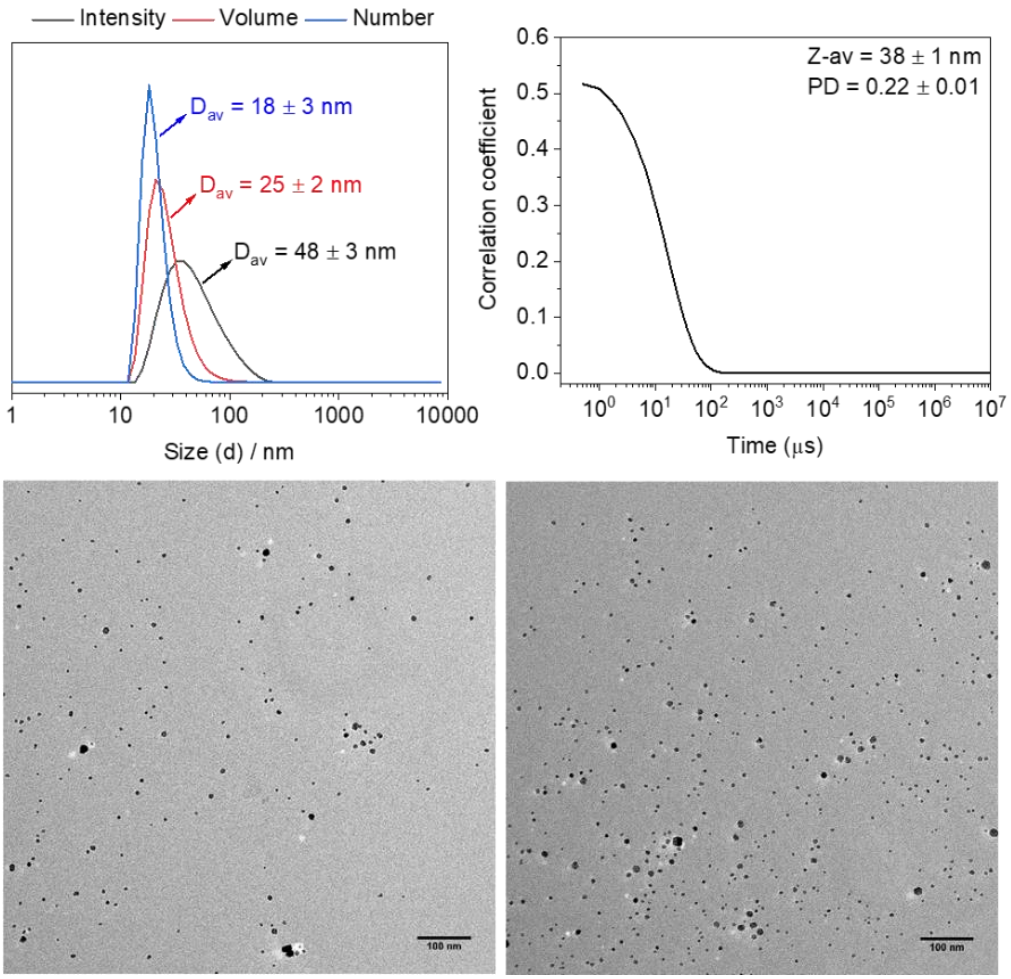


Figure S16. DLS size distributions (top left), DLS correlation function (top right) in hexane and TEM images of IONPs (bottom).

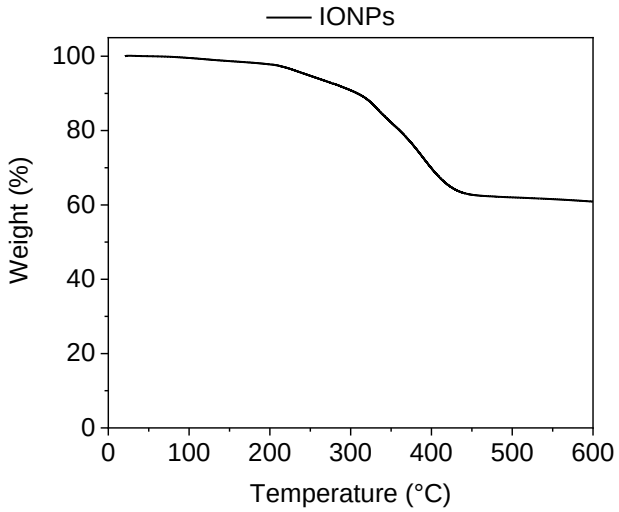


Figure S17. TGA thermogram of polymer free IONPs.

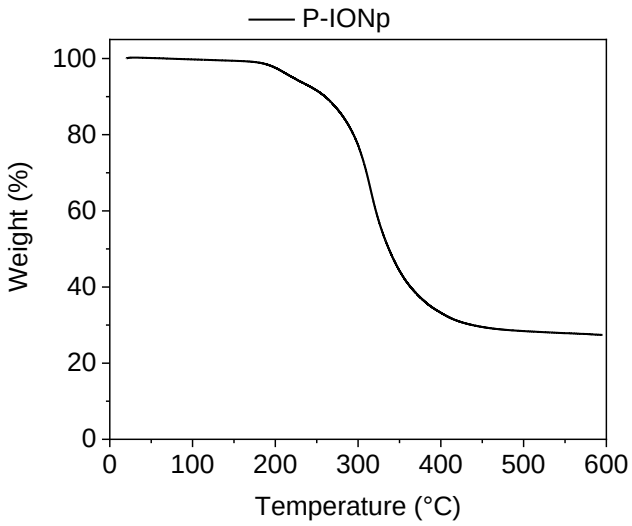


Figure S18. TGA thermogram of **P-IONp**.

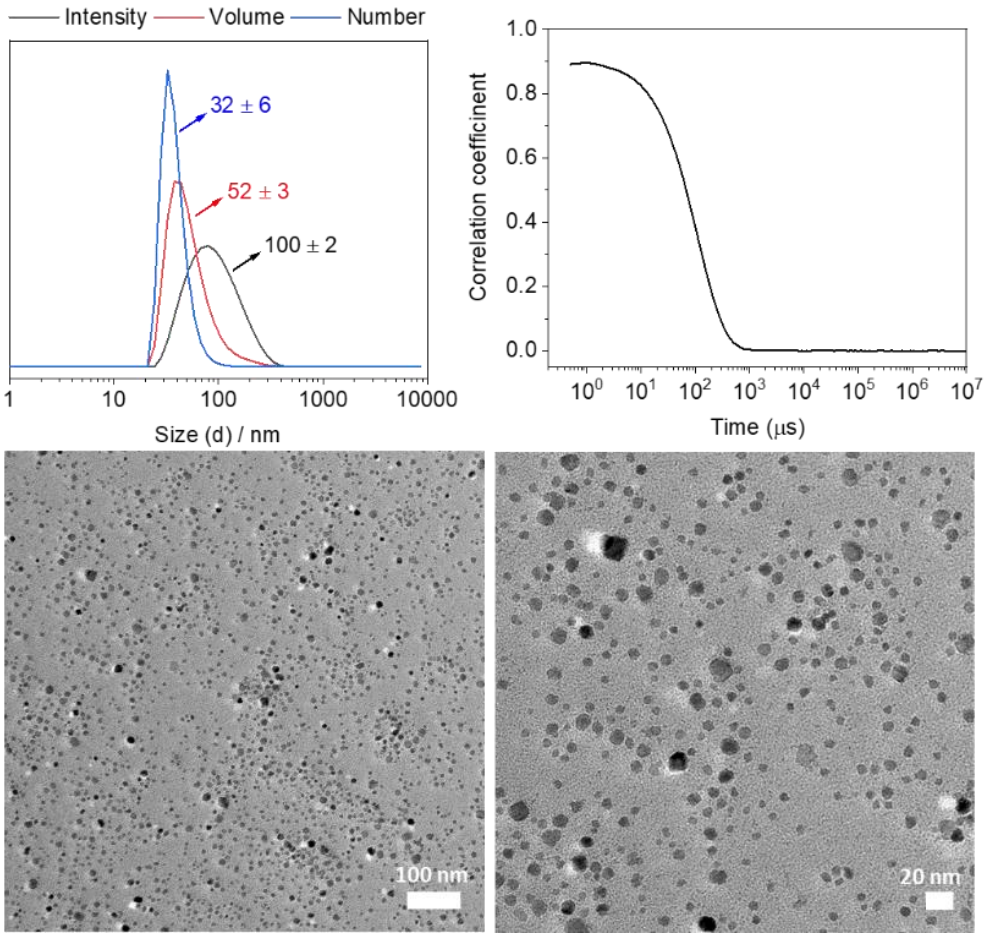


Figure S19. DLS size distributions (top left), DLS correlation function (top right) in water and TEM images of **P-IONp** (bottom).

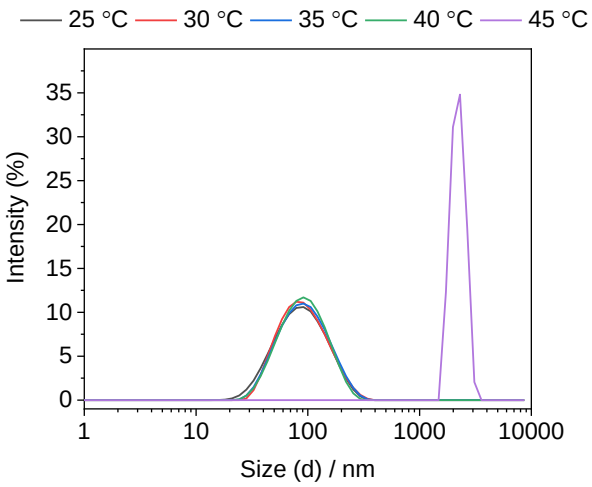


Figure S20. DLS size distributions of **P-IONp** in water at 2 mg mL^{-1} as a function of the temperature.

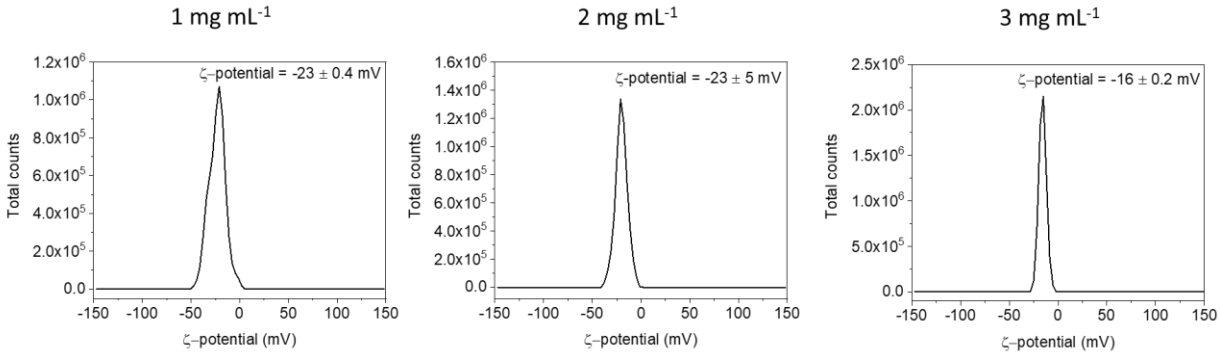


Figure S21. ζ-potential distributions of the hybrid nanogels synthesized with an initial **P-IONp** concentration of 1, 2 and 3 mg mL^{-1} in pure water.

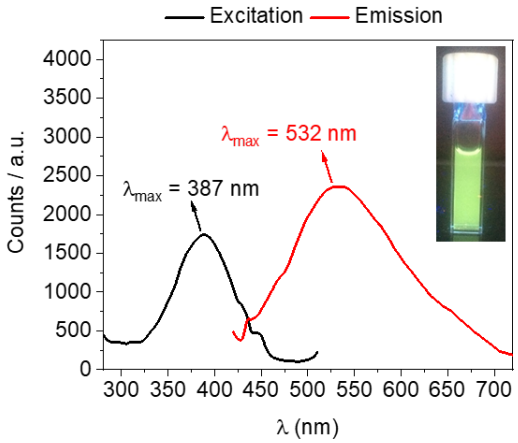


Figure S22. Excitation and emission spectra of hybrid nanogels in water synthesized with an initial polymer grafted iron oxide nanoparticle concentration of 2 mg mL^{-1}.

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