Supporting Information for

Catalytic Single-Chain Polymeric Nanoparticles at Work:
From Ensemble towards Single-Particle Kinetics

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1. Materials and Methods

Rhodamine 110 chloride (HCl salt of Rhodamine, 99%) was purchased from Sigma-Aldrich. Triphosgene (Sigma-Aldrich, 98%), propargyl chloroformate (Sigma-Aldrich, 96%), 2-methylbut-3-yn-2-ol (Sigma-Aldrich, 98%), 4-morpholinecarbonyl chloride (Sigma-Aldrich, 98%), NaH (Sigma-Aldrich, 60% dispersion in mineral oil), but-2-yn-1-ol (Sigma-Aldrich, 98%), prop-2-en-1-ol (Sigma-Aldrich, >99%), PyBOP (Sigma-Aldrich, 98%), 5-((tert-butoxycarbonyl)amino)pentanoic acid (Sigma-Aldrich, 97%), 1,10-phenanthrolin-5-amine (Sigma-Aldrich, 97%). Azobisisobutyronitrile (AIBN) was recrystallized from methanol, 4-cyano-4-((phenylcarbonothioyl)thio)pentanoic acid was kindly provided by SyMO-Chem (Eindhoven, the Netherlands). Pentafluorophenyl acrylate (Sigma-Aldrich, 98%), Jeffamine® M-1000 (Jeffamine M1000, PO/EO mol ratio = 3/19, Lot n$^{\circ}$: 0L504, Huntsman Holland BV), 1,4-dioxane (Sigma-Aldrich, anhydrous, 99.8%), copper(II) sulfate pentahydrate (Sigma-Aldrich, $\geq$ 98.0%), sodium ascorbate (Sigma-Aldrich, $\geq$ 98.0%), lauroyl peroxide (Sigma-Aldrich, 97%), biotin-PEG-amine (poly(ethylene glycol) 2-aminoethyl ether biotin, Sigma-Aldrich, average M_n~2300), streptavidin (Sigma-Aldrich) were used as received. BTA-amine was synthesized according to a previously reported procedure.[1] 3-Aminopropyl dimethylethoxy silane was obtained from ABCR. Biotin-PEG-NHS (MW = 3000 Da) was purchased from RAPP Polymere. ATTO488 carboxylic acid was purchased from ATTOTEC, Alexa Fluor® 488 Cadaverine was purchased from ThermoFisher. Column purifications were done with th eBiotage Isolera one using KP-SIL SNAP cartridges (flash silica) as the stationary phase. Dialysis was performed using molecular porous membrane tubing from Spectra/Por®dialysis with a MWCO of 6-8000.

2. Characterization methods

Ultraviolet-visible (UV/Vis) and circular dichroism (CD) measurements were performed on a Jasco J-815 spectropolarimeter where the sensitivity, time constant and scan rate were chosen appropriately. Corresponding temperature-dependent measurements were performed with a PFD-425S/15 Peltier-type temperature controller with a temperature range of 263-383 K and adjustable temperature slope, in all cases a temperature slope of 1 K/min was used. Fluorescence measurements were performed on a Jasco FP-6500 spectrofluorometer. Dynamic light scattering measurements were performed on a Malvern mV Zetasizer equipped with an 830 nm laser. Samples were prepared in MilliQwater, followed by ultrasonfication for 45 mins,

then put into a preheated oven at 90 °C for 45 mins, then the heating was turned off and the samples were left to rest overnight. Before measuring, the solutions were filtered through a 0.1 µm PVDF-filter. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Varian Mercury Vx 400 MHz, where chemical shifts were determined with respect to tetramethylsilane (TMS) as an internal reference. DMF-SEC measurements were carried out in PL-GPC-50 plus from Polymer Laboratories (Agilent Technologies) with the refractive index detector working in DMF containing 10 mM LiBr at 50 °C at a constant flow rate of 1 mL min^{-1} on a Shodex GPC-KD-804 column (exclusion limit = 400 000 Da; 0.8 cm i.d. × 300 mL), which was calibrated with polyethyleneoxide (PEO) samples with a range from 282-77350 Da (Polymer Laboratories-Agilent Technologies). THF-SEC-measurements were performed on a Shimadzu-system with two Agilent Technology columns in series (PLgel 5 mm mixed C [200– 2 000 000Da] and PLgel 5 mm mixed D [200–40 000 Da]) and equipped with a RI detector (Shimadzu RID-10A) and a PDA detector (Shimadzu SPD-M10A), with THF as eluent at a constant flowrate of 1.0 mL min^{-1}. The system was calibrated with polystyrene (PS) samples with a range of 580–100 000 Da (Polymer Laboratories). LC-MS experiments were performed on ThermoScientific LCQ Fleet instrument. Gradient with Water and Acetonitrile (with 0.1% formic acid). The conversion of reactions were calculated by comparing the integration of substate's retention peak with the calibration.

3. Synthetic procedures

Synthesis of pPFPA:

The synthesis of pPFPA was following the similar procedure reported previously. [i]

A Schlenk tube was charged with a stir bar, pentafluorophenylacrylate (4.41 g, 18.53 mmol, 223 eq.),4-cyano-4-((phenylcarbonothioyl)thio)pentanoic acid (23.24 mg, 0.083 mmol, 1.0 eq.), azobis-isobutyronitrile (AIBN, 1 mg, 6.1 µmol, 0.07 eq.), and dioxane (4.0 mL). The solution was degassed by gently bubbling argon through the solution for 30 minutes. Subsequently, the Schlenk tube was placed into a preheated oil bath at 80 °C. After 2 hours 30 minutes, the polymerization was quenched by placing the Schlenk tube in a liquid-nitrogen bath. The monomer conversion was determined to be 53 % by measuring ^{19}F NMR of the reaction mixture. The degree of polymerization (*DP*) was then estimated to be 120. The formed polymer was isolated by precipitation in pentane (three times) and by drying under vacuum to yield a slightly pink powder. The phenylcarbonothioylthio end group of the polymer was

removed through the following procedure. The obtained polymer was dissolved in 5 mL dioxane together with 273 mg AIBN and 64 mg of lauroyl peroxide. The mixture was degassed via bubbling argon for 30 mins and subsequently put into a preheated oil bath at 80 °C. After 4 hours, the pink color of the mixture turned to be colorless. The reaction was quenched by immersing the flask in liquid nitrogen. The final polymer pPFPA was obtained by precipitating the reaction mixture in pentane for 3 times as a white powder. ^{1}H-NMR (400 MHz, CDCl3): δ 3.08 (br), 2.49 (br), 2.11 (br); ^{19}F-NMR: δ -153.2 (br), -156.8 (br), -162.2 (br), GPC (THF): M_n = 18.0 kDa, $Đ$ = 1.28.
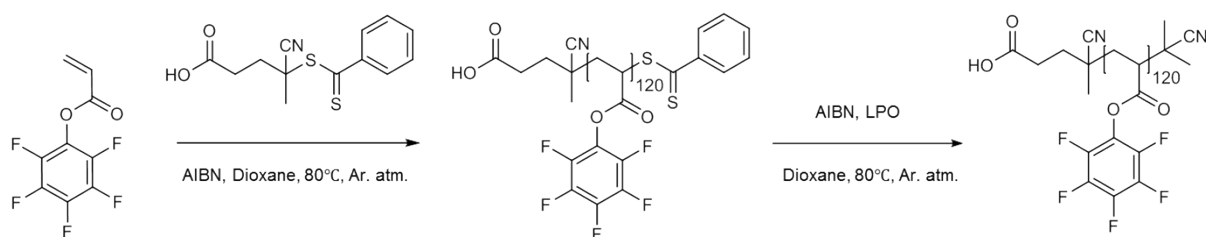
Fig. S1: Synthesis of the precursor polymer **pPFPA**.

Synthesis of Phen-C4-NH$_2$:
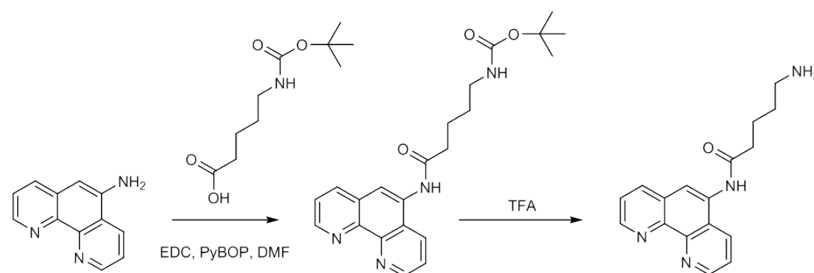
Phen-C4-NHBoc: 1.24 g PyBOP and 520 mg 5-((tert-butoxycarbonyl)amino)pentanoic acid were put into 100 mL flask and dissolved in 30 mL DMF. The mixture was stirred at room temperature for 30 mins under argon. Then, 390 mg 1,10-phenanthrolin-5-amine and 1.5 mL DIPEA were added in one portion. After another 30 min stirring at room temperature, the flask was put into pre-heated oil bath at 40 °C for 72 h. The DMF was removed by evaporation under vacuum. The residue was purified using column chromatography with methanol:CHCl$_3$ (3/97 v/v). Yield 0.21g, 27%. ^{1}H-NMR (400 MHz, CDCl$_3$): δ = 9.23 (q, 1H, aromatic), 9.14 (d, 1H, aromatic), 8.48 (d, 1H, aromatic), 8.34 (s, 1H, aromatic), 7.70 (q, 1H, aromatic), 7.62 (q, 1H, aromatic), 4.74 (s, 1H, CO-NH), 3.29 (2H, -CH$_2$-), 2.66 (2H, -CH$_2$-), 1.91 (2H, -CH$_2$-), 1.67 (2H, -CH$_2$-), 1.32 (9H, -CH$_3$). LC-MS Calcd. [M+H]$^+$= 395.20, Obs. [M+H]$^+$= 395.25.

Phen-C4-NH₂: 16 mg Phen-NHBoc was dissolved in 2 mL CHCl$_3$, then 2 mL TFA was added. The mixture was stirred at room temperature for 1 hour. LC-MS confirmed full deprotection. The solvent was removed by high vacuum. The formed Phen-C4-NH$_2$ was used without further purification. LC-MS Calcd. $[M+H]^+= 295.15$, Obs. $[M+H]^+= 295.25$.

Synthesis of P1-P3:

120 mg pPFPA was dissolved in 4 mL DMF and added into the Phen-NH$_2$ containing flask (after Phen-NHBoc 16 mg / TFA reaction). Then, 30 mg DIPEA was added to neutralize the TFA. The mixture was heated at 50 °C for 4 h, ^{19}F-NMR confirmed full conversion. 33 mg BTA-amine was dissolved in 3 mL DMF and added into the flask for the modification. After 2 h reaction at 50 °C, ^{19}F-NMR confirmed full conversion. The solvent was then removed and the residue was re-dissolved in 6 mL DMF and divided into 3 fractions.

P1: 3 mL of the solution was moved to another flask, 600 mg Jeffamine in 2 mL DMF was added. The mixture was heated at 50 °C overnight to reach full modification.

P2: 1.5 mL of the solution was moved to another flask, 2.9 mg Biotin-PEG-amine in 2 mL DMF was added. The mixture was heated at 60 °C for 2 hours. After ^{19}F-NMR test, 350 mg Jeffamine was added and reacted at 50 °C overnight to reach full modification.

P3: 1.5 mL of the solution was moved to another flask, 2.9 mg Biotin-PEG-amine and 0.8 mg Alexa Fluor 488 amine in 2 mL DMF was added. The mixture was heated at 60 °C for 2 hours. After ^{19}F-NMR test, 350 mg Jeffamine was added and reacted at 50 °C overnight to reach full modification.

Full conversion of all the three reactions were confirmed by ^{19}F-NMR. The reaction mixtures were directly put into dialysis tubes individually, against THF for 2 days then methanol for 2 days, to obtain the final polymers.

Note: The precursor polymer was observed to be slightly hydrolyzed during the modification procedure, possibly due to the heating and addition of the base DIPEA. The ratio of hydrolysis was calculated through ^{19}F NMR.
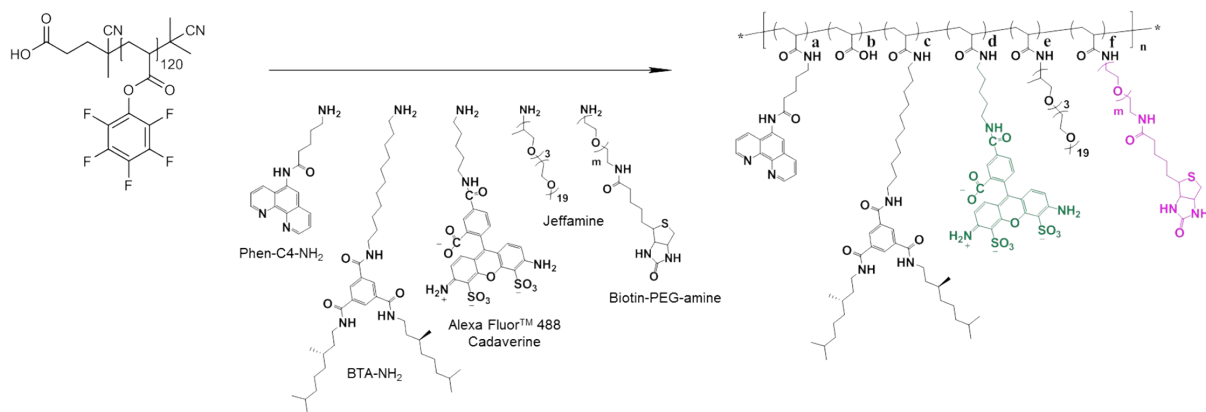
Fig. S2: Synthesis of the polymers via post-polymerization modification of **pPFPA**.
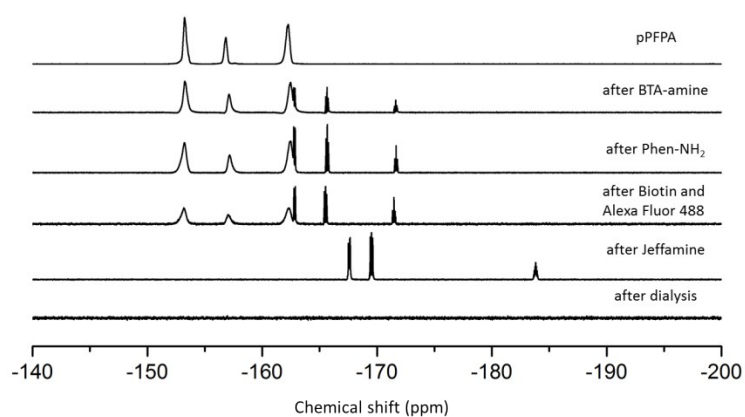
Fig. S3: Monitoring of the polymer analogue reactions by ^{19}F-NMR.

(Synthesis of **P3** from **pPFPA** was chosen as an example here)
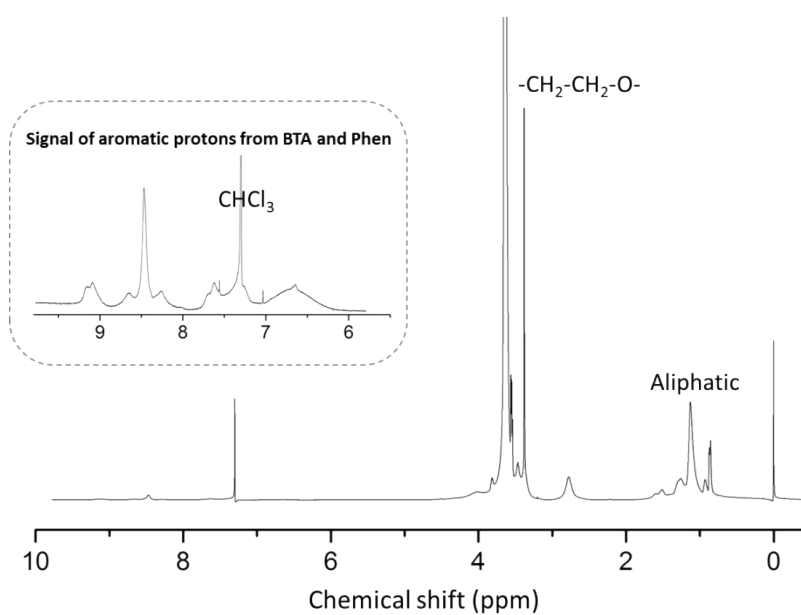

Fig. S4 : ^{1}H-NMR of **P3** as a representative example.

Synthesis of P4:

40 mg pPFPA was dissolved in 2 mL DMF, then 11 mg BTAamine and 2.5 mg dodecylamine were dissolved in 2 mL DMF together and added into the pPFPA solution. The mixture was heated at 50 °C for 2 hours, ^{19}F-NMR confirmed full conversion. 350 mg Jeffamine was dissolved in 3.5 mL DMF and added into the flask, the mixture was stirred 50 °C overnight. ^{19}F-NMR confirmed full conversion. The reaction mixture was directly put into dialysis tube and dialysed against THF for 2 days then methanol for 2 days, to obtain the final polymers. ^{1}H-NMR (400 MHz, CDCl3): δ 8.6-8.4 (aromatic: BTA), 7.0-6.0 (-NHCO-), 4.3-4.1 (-CO$_2$CH$_2$CH$_2$-), 4.2-3.0 (-NHCOC$\underline{H}$CH$_2$-,-OC$_2$H$_4$O-, -OCH$_3$), 2.8-2.5 (-CO$_2$CHC$\underline{H}_2$-), 1.6-0.8 (-OCHC$\underline{H}_3$, aliphatic: BTA and dodecyl). GPC(DMF): Mn = 31.7 kDa, Đ = 1.20.

Synthesis of Substrate S1-S4:

Rhodamine 110 → (a) → MCR 110 → (b) S1, (c) S2, (d) S3, (e) S4
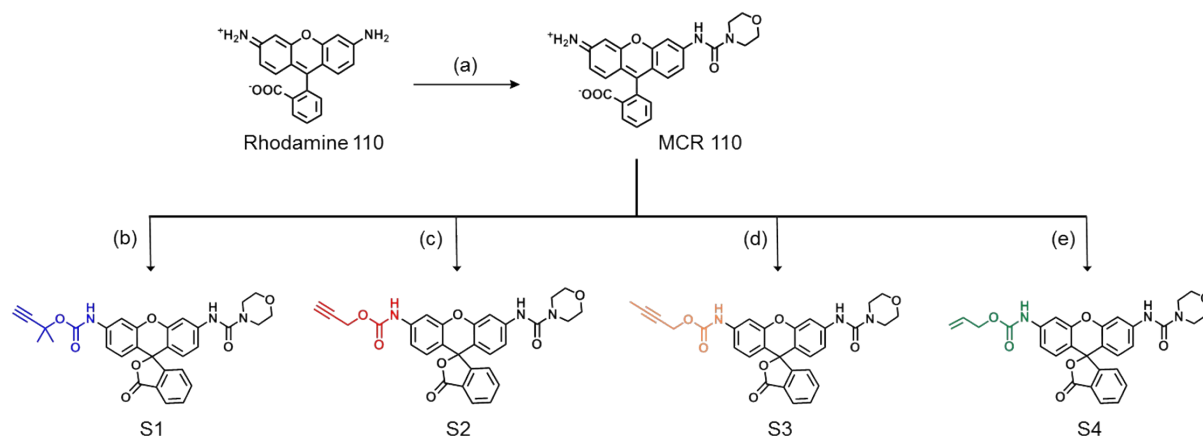
MC-Rh 110: 532 mg Rhodamine110 chloride was dissolved in 50 mL DMF in a nitrogen gas atmosphere. Then 117 mg of a NaH dispersion (60% dispersion in mineral oil; 70.2 mg NaH) was added portion wise during 8 minutes. After stirring for 55 minutes, 170 μL of 4-morpholinecarbonyl chloride was dropwise added during 5 minutes. The reaction was covered by aluminium foil to exclude light and stirring was continued overnight. The reaction mixture was then concentrated on a rotary evaporator with high vacuum. The desired compound was obtained after column chromatography with CHCl$_3$/methanol/acetic acid 100/7/1 v/v/v mixture as eluent. Yield 155 mg, 25.6%. ^{1}H-NMR (400 MHz, CDCl$_3$): δ = 7.97 (m, 1H), 7.60 (m, 2H), 7.42 (d, 1H), 7.28 (s, 1H), 7.12 (d, 1H), 6.84 (q, 1H), 6.50 (q, 2H), 6.43 (d, 1H), 6.30 (q, 1H), 3.63 (4H), 3.44 (4H). ^{13}C NMR (400 MHz, CDCl$_3$): δ = 170.19, 154.97, 152.74, 151.88, 149.28, 141.43, 135.09, 129.66, 128.96, 128.08, 126.91, 124.88, 124.27, 115.50, 112.99,

111.69, 108.13, 107.58, 101.41, 66.47, 44.25. LC-MS Calcd. [M+H]$^+$= 444.15, Obs. [M+H]$^+$= 444.33.

S1: Triphosgene 640 mg was added into a two neck oven-dried flask, then was dissolved in a mixture of 2 mL acetronitrile and 1 mL CHCl$_3$. The flask was put in an ice bath and cooled. Under argon flow, 120 mg MC-Rh 110 in 2 mL acetonitrile was added dropwise. Orange precipitates formed immediately. The mixture was stirred for 1 h. 1.64 g 2-methylbut-3-yn-2-ol and 1.6 mL pyridine were mixed and added into the flask. The precipitates dissolved again, which resulted in a red, transparent solution. Stiring was continued at room temperature for another 2 h, LC-MS confirmed the formation of **S1**. The solvent was removed via evaporation in vacuo. The residue was dissolved in 40 mL CHCl$_3$, then washed with diluted HCl (1N), sat. NaHCO$_3$ and brine. The organic phase was dried over Na$_2$SO$_4$, filtered and evaporated in vacuo. The product was obtained after column chromatography (eluent: gradient from pure CHCl$_3$ to 50:50 v/v CHCl$_3$: EtOAc). Yield 50 mg, 33%. ^{1}H-NMR (400 MHz, DMSO): δ = 9.92 (s, 1H), 8.85 (s, 1H), 8.01 (d, 1H), 7.76 (m, 2H), 7.65 (d, 1H), 7.57 (d, 1H), 7.27 (d, 1H), 7.15 (m, 2H), 6.66 (q, 2H), 3.61 (t, 4H), 3.56 (s, 1H), 3.44 (t, 4H), 1.69 (s, 6H). LC-MS Calcd. [M+H]$^+$= 554.18, Obs. [M+H]$^+$= 554.08.
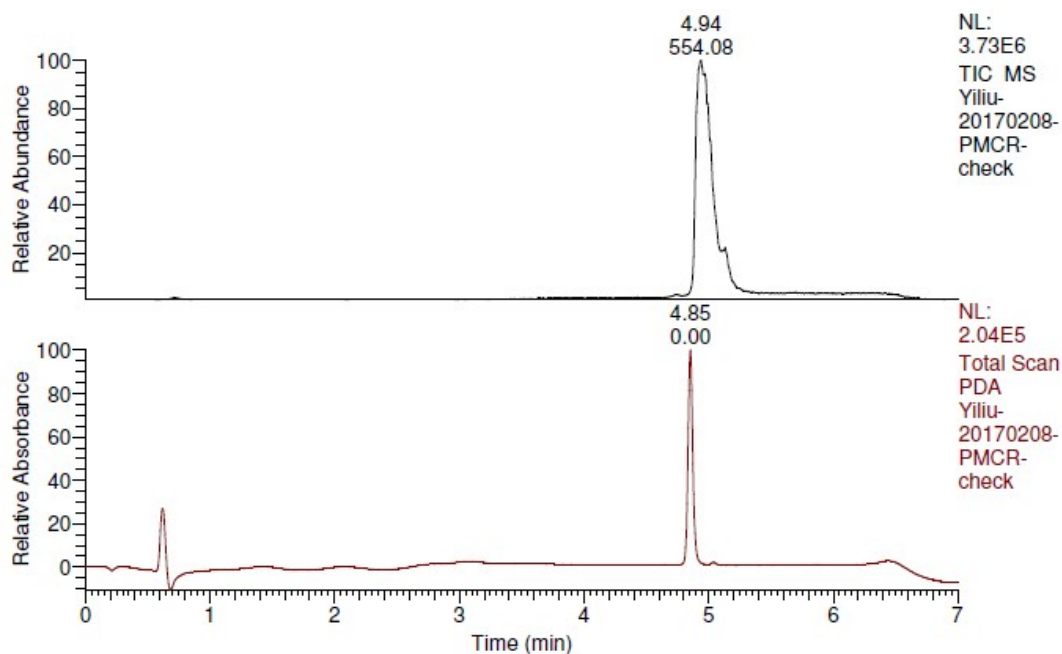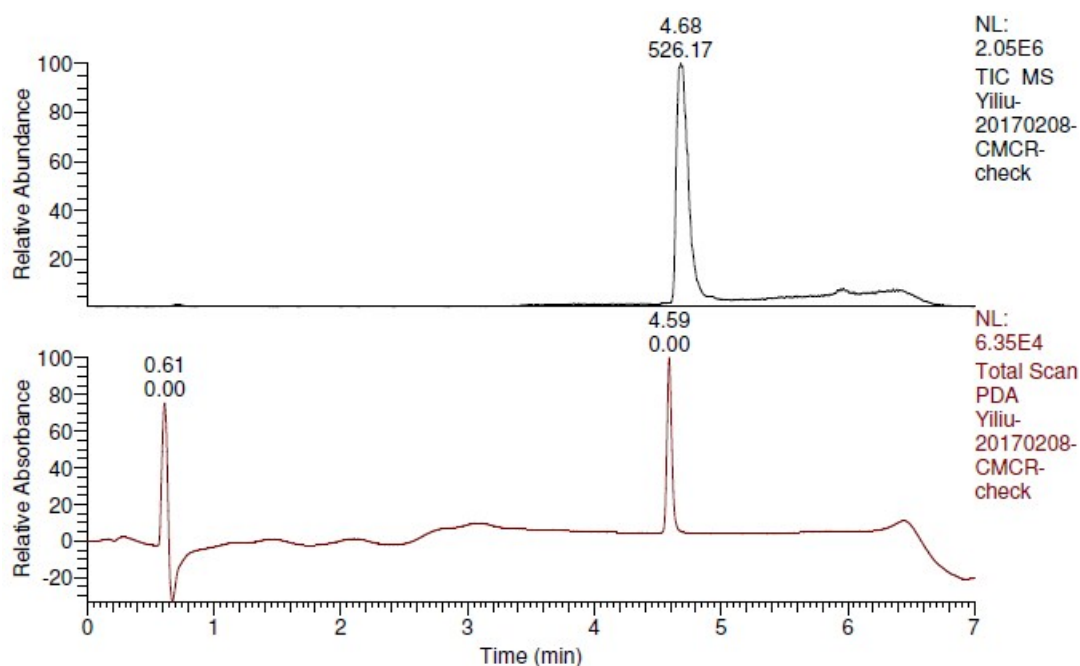

LC-MS analysis of **S1**

S2: MC-Rh 110 (190 mg) was weighed into a round-bottom flask and dissolved in 6 mL dry DMF. Subsequently, 2 mL of dry pyridine was added. The flask was sealed with a septum and

an argon filled balloon was attached. After cooling in an ice/water bath, 0.40 ml of propargyl chloroformate was added dropwise through a syringe. The mixture was stirred at room temperature overnight. The solvent was removed via evaporation in vacuo. The residue was dissolved in 100 mL CHCl₃, washed with diluted HCl (1N), water and brine. The organic phase was dried over MgSO₄. The product was obtained after column chromatography (eluent: EtOAc). Yield 50 mg, 21%. %. ¹H-NMR (400 MHz, CDCl₃): δ = 7.97 (m, 1H), 7.60 (m, 2H), 7.51 (s, 1H), 7.35 (m, 2H), 7.01-7.05 (m, 3H), 6.93 (q, 1H), 6.68 (d, 2H), 6.59 (d, 1H), 4.77 (d, 2H), 3.70 (4H), 3.50 (4H), 2.51 (t, 1H). LC-MS Calcd. [M+H]⁺= 526.15, Obs. [M+H]⁺= 526.17.

LC-MS analysis of **S2**

S3: Triphosgene (500 mg) was added into a two neck oven-dried flask, and dissolved in a mixture of 2 mL acetronitrile and 1 mL CHCl₃. The mixture was cooled in an ice bath. Under argon flow, 70 mg MC-Rh 110 and 15 mg dry pyridine were mixed in 2 mL acetonitrile, and added dropwise to the flask. An orange precipitate was immediately formed. The mixture was stirred for another 1 h. Then, 700 mg but-2-yn-1-ol and 1.0 mL pyridine were mixed in 1 mL acetonitrile and added into the flask. The precipitate dissolved again resulting in an orange/red transparent solution. The mixture was stirred at room temperature for another 2 h, after which LC-MS confirmed the formation of S3. The solvent was removed via evaporation in vacuo. The residue was dissolved in 50 mL CHCl₃, then washed with diluted HCl (1N), sat. NaHCO₃ and brine. The organic phase was dried by Na₂SO₄, filtered and evaporated in vacuo. The

product was obtained after column chromatography (eluent: gradient form pure $CHCl_3$ to 50:50 v/v $CHCl_3$: EtOAc). Yield 48 mg, 56%. ^{1}H-NMR (400 MHz, DMSO): δ = 10.13 (s, 1H), 8.84 (s, 1H), 8.01 (d, 1H), 7.76 (m, 2H), 7.67 (d, 1H), 7.55 (d, 1H), 7.28 (d, 1H), 7.14 (m, 2H), 6.66 (q, 2H), 4. 75 (q, 2H), 3.61 (t, 4H), 3.44 (t, 4H), 1.85 (t, 3H). LC-MS Calcd. [M+H]$^+$= 540.17, Obs. [M+H]$^+$= 540.33.
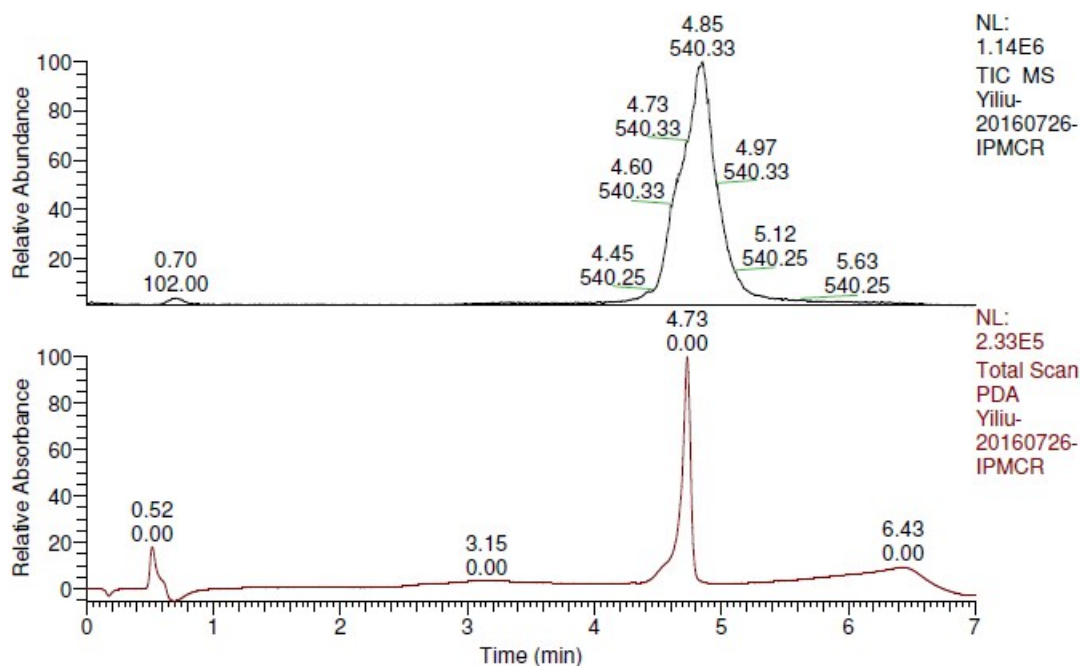
LC-MS analysis of **S3**

S4: Triphosgene (540 mg) was added into a two neck oven-dried flask, and dissolved in a mixture of 2 mL acetronitrile and 1 mL $CHCl_3$. The mixture was cooled with an ice bath, and under argon flow, 80 mg MC-Rh 110 and 15 mg dry pyridine were dissolved in 2 mL acetonitrile were added dropwise. An orange precipitate was immediately formed. The mixture was stirred for another 1 h. Then 1.5 mL prop-2-en-1-ol and 1.0 mL pyridine were mixed in 1 mL acetonitrile and added into the flask. The precipitate dissolved again, resulting in a red, transparent solution. Stirring was continued at room temperature for another 4 h, after which LC-MS confirmed the formation of **S4**. The solvent was removed via evaporation in vacuo. The residue was dissolved in 50 mL $CHCl_3$, then washed with diluted HCl (1N), sat. $NaHCO_3$ and brine. The combined organic phase was dried by Na_2SO_4, filtered and evaporated in vacuo. The product was obtained after column chromatography (eluent: 50:50 v/v $CHCl_3$: EtOAc). Yield 38 mg, 40%. ^{1}H-NMR (400 MHz, DMSO): δ = 10.06 (s, 1H), 8.84 (s, 1H), 8.01 (d, 1H), 7.76 (m, 2H), 7.66 (d, 1H), 7.57 (d, 1H), 7.27 (d, 1H), 7.15 (m, 2H), 6.66 (q, 2H), 5.99 (m, 1H),
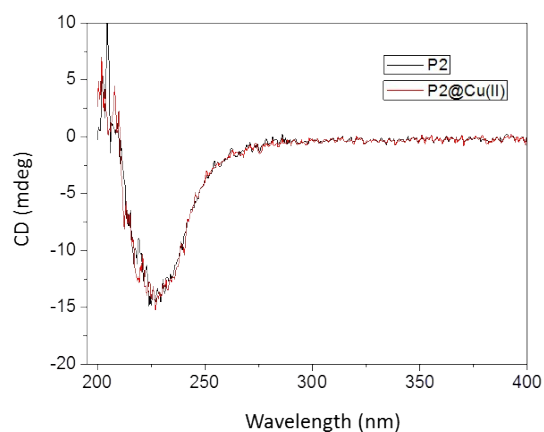
5.36 (q, 1H), 5.25 (q, 1H), 4. 63 (m, 2H), 3.61 (t, 4H), 3.44 (t, 4H), 1.69 (s, 6H). LC-MS Calcd. [M+H]$^+$= 528.17, Obs. [M+H]$^+$= 528.25.
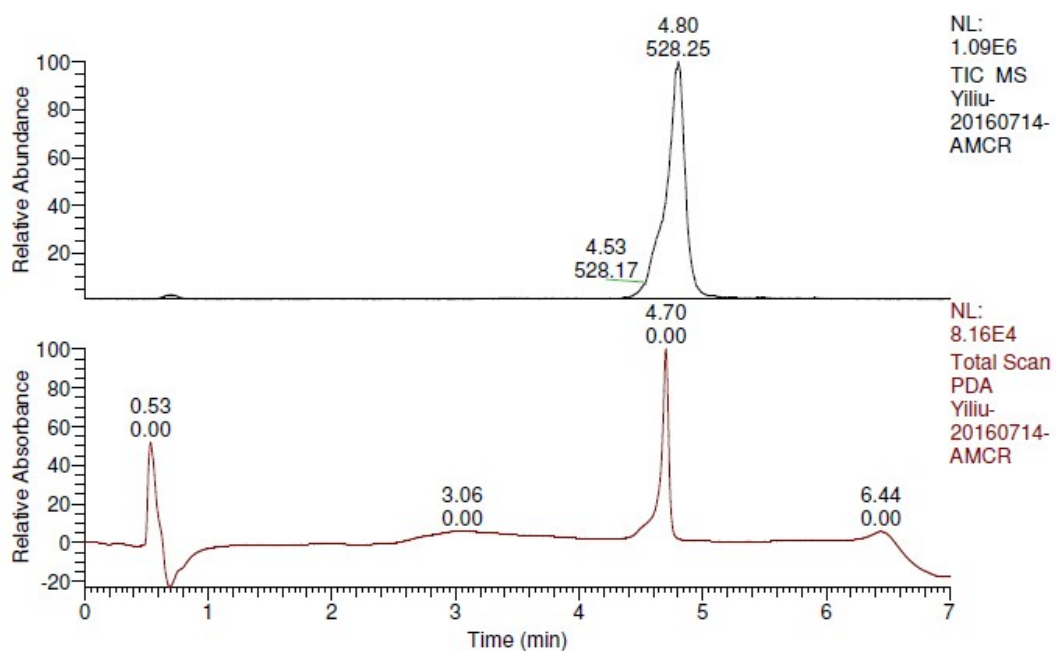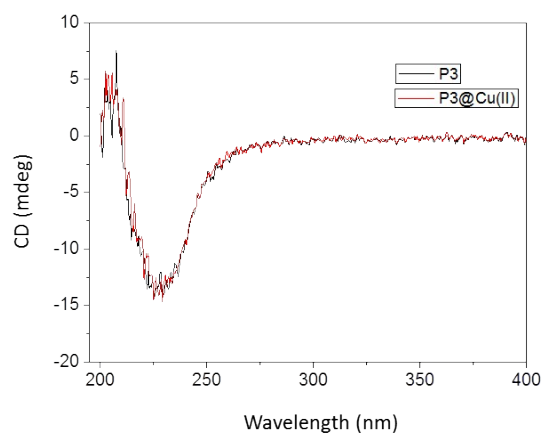

LC-MS analysis of **S4**

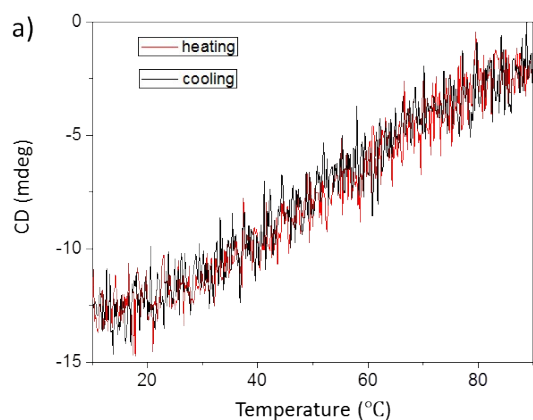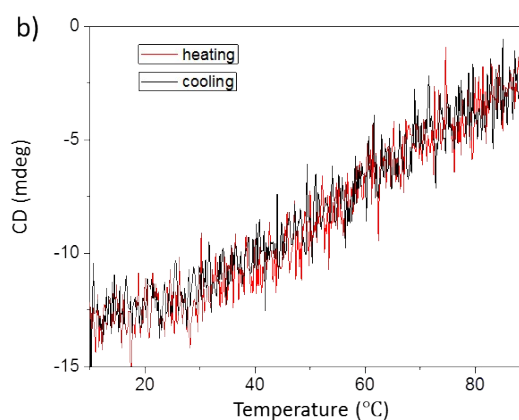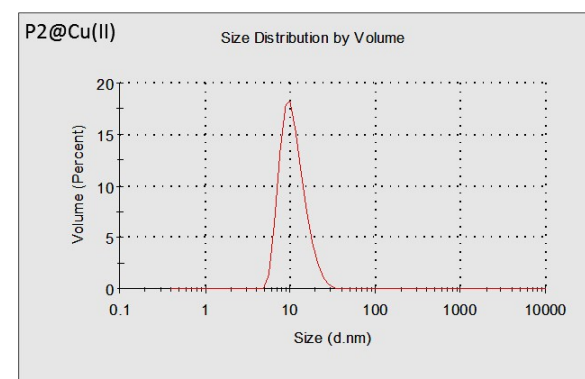## 4. Spectroscopic and scattering experiments

CD measurements:

(A)

(B)

(C)

(D)

Fig. S5: CD spectra of the polymers **P2** and **P3** before (A) and after (B) loading with $CuSO_4$ (0.5 mg/mL; 0.5 cm cuvette; Phen:Cu=2:1). Cooling and heating CD curves of (C) **P1** (a; 0.5 mg/mL) and (D) **P1@Cu(II)** (b; Phen:Cu=2:1). Temperature from 10 ℃ to 90 ℃, monitored at 223 nm (cooling and heating rate at 1 K/min).

Scattering measurements:
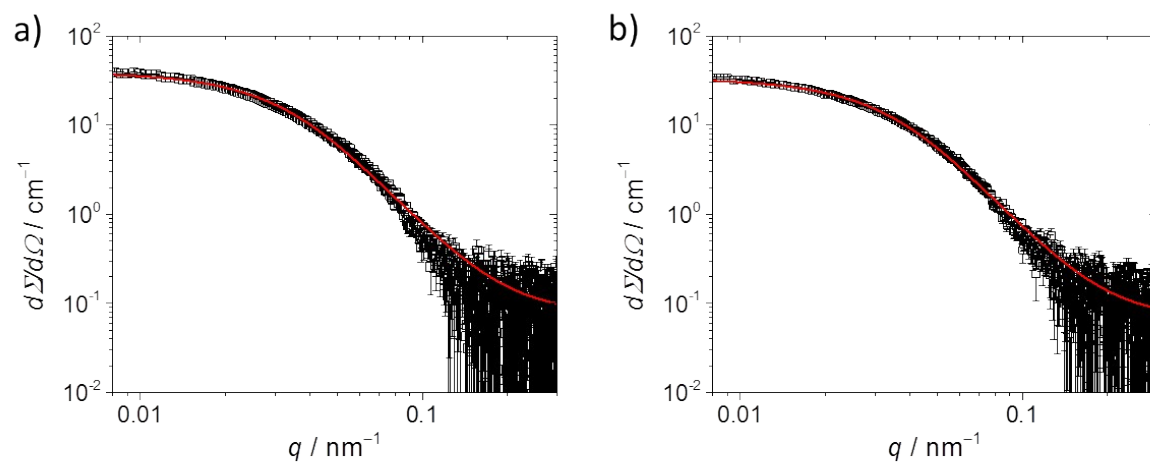
Fig. S6: SAXS measurements (a) **P1** (1 mg/ml); (b) **P1@Cu(II)** (1 mg/ml).

P1 — Size Distribution by Intensity

P1 — Size Distribution by Volume

P1@Cu(II) — Size Distribution by Intensity

P1@Cu(II) — Size Distribution by Volume

P2 — Size Distribution by Intensity

P2 — Size Distribution by Volume

P2@Cu(II) — Size Distribution by Intensity

P2@Cu(II) — Size Distribution by Volume
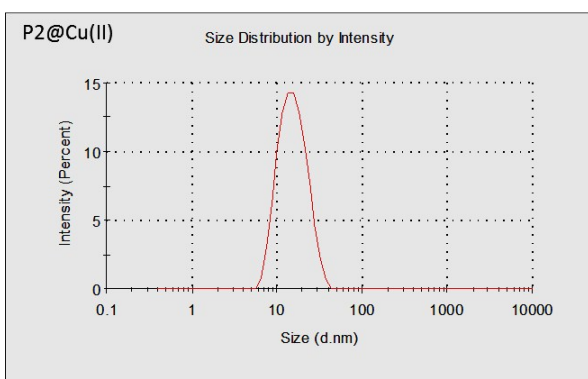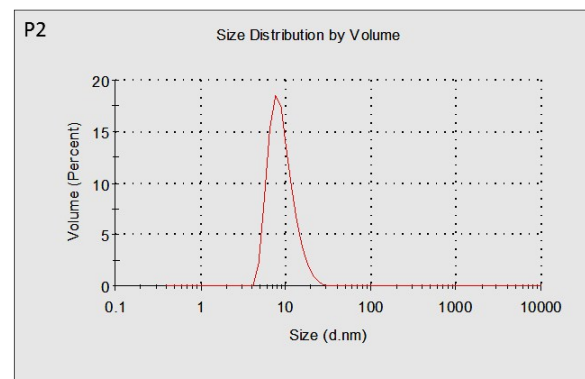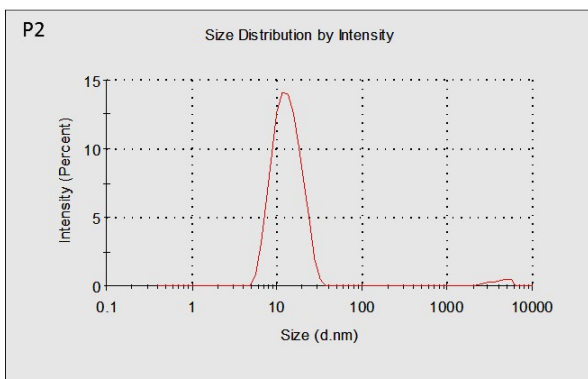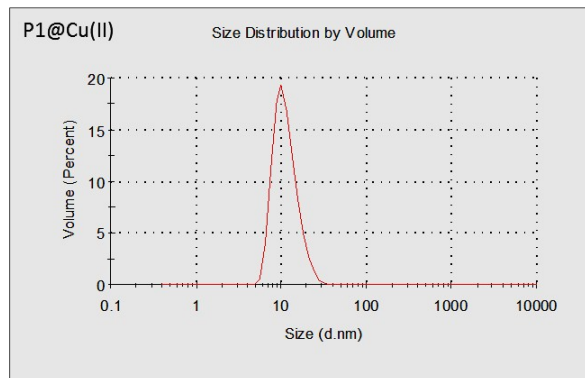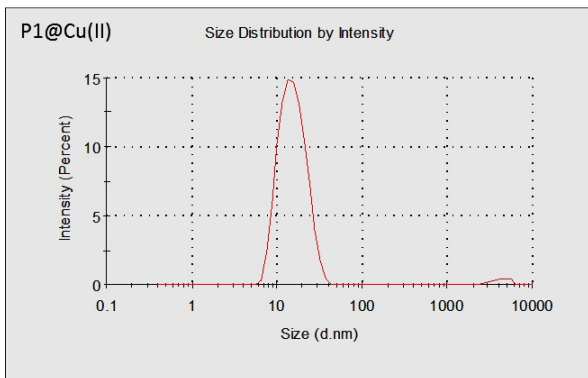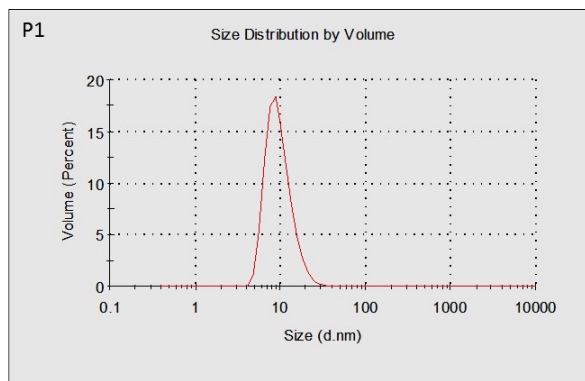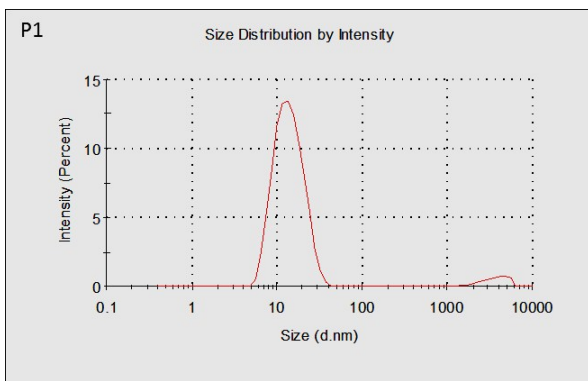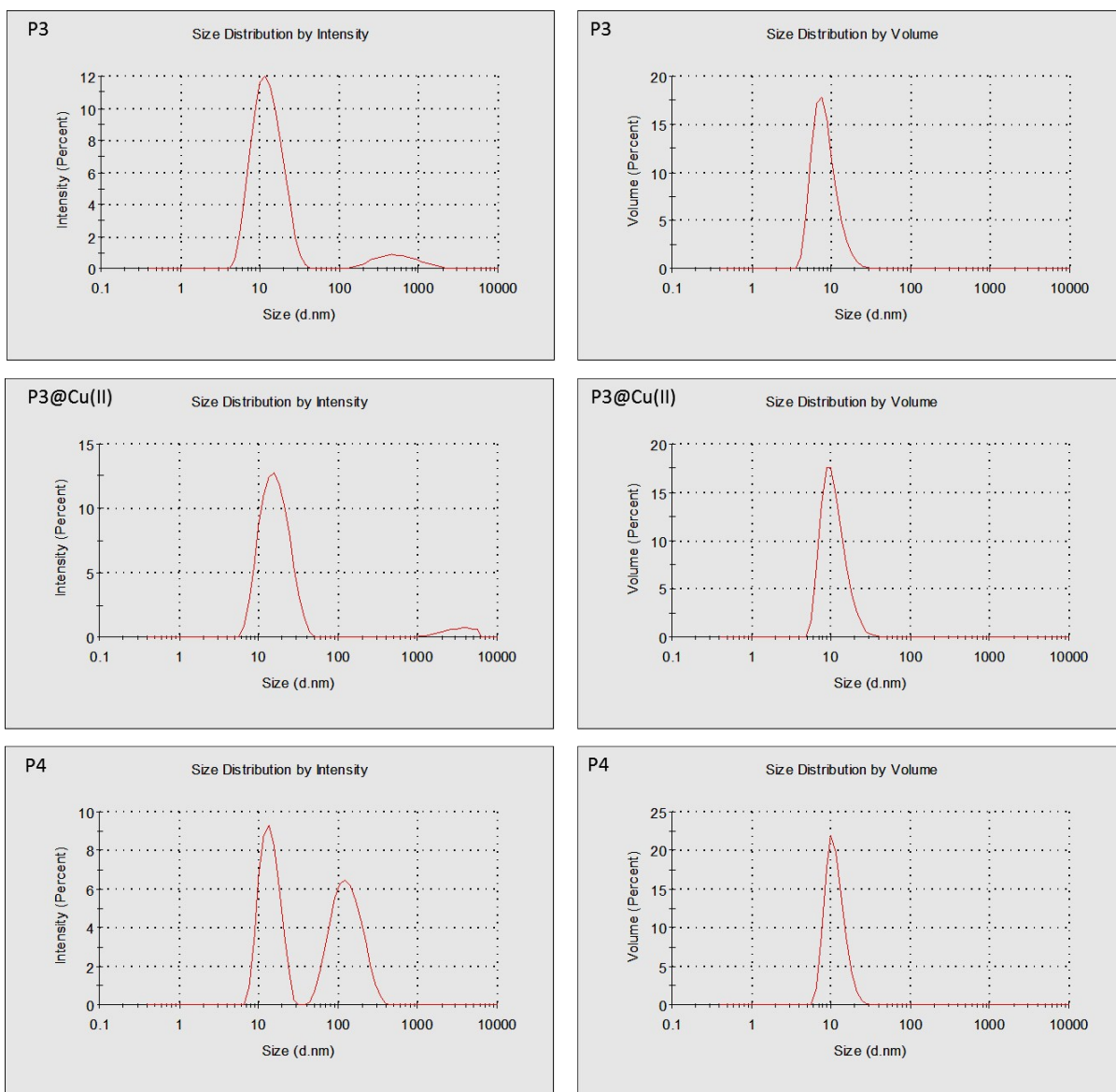
Fig. S7: DLS results (by intensity and volume)of the polymers (1 mg/mL) before and after loading $CuSO_4$ (phen:Cu=2:1).

Table S1: DLS results of the polymer nanoparticles in aqueous solution.

Sample	Size by Intensity (R_h, nm)	Size by Number (R_h, nm)
P1	7.42	5.04
P1@Cu(II)	8.22	5.61
P2	6.89	4.65
P2@Cu(II)	7.86	5.72
P3	6.60	4.36
P3@Cu(II)	8.39	5.59
P4	7.12	5.68

Fluorescence correlation spectroscopy:
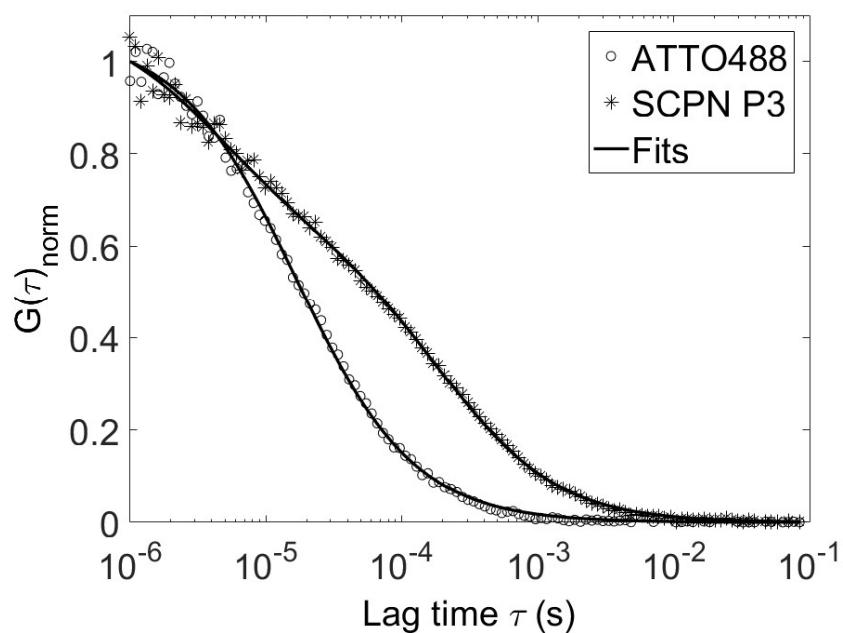
Fig. S8: Example autocorrelation functions showing the diffusion of ATTO488 and the Alexa Fluor® 488 labeled SCPN **P3.** The solid lines represent the fits: a one-component 2D-diffusion model for ATTO488 and a two-component 2D-diffusion model for SCPN **P3**. Details of the followed procedures can be found in section 6.

5. Catalysis experiments

Carbamate cleavage reactions:
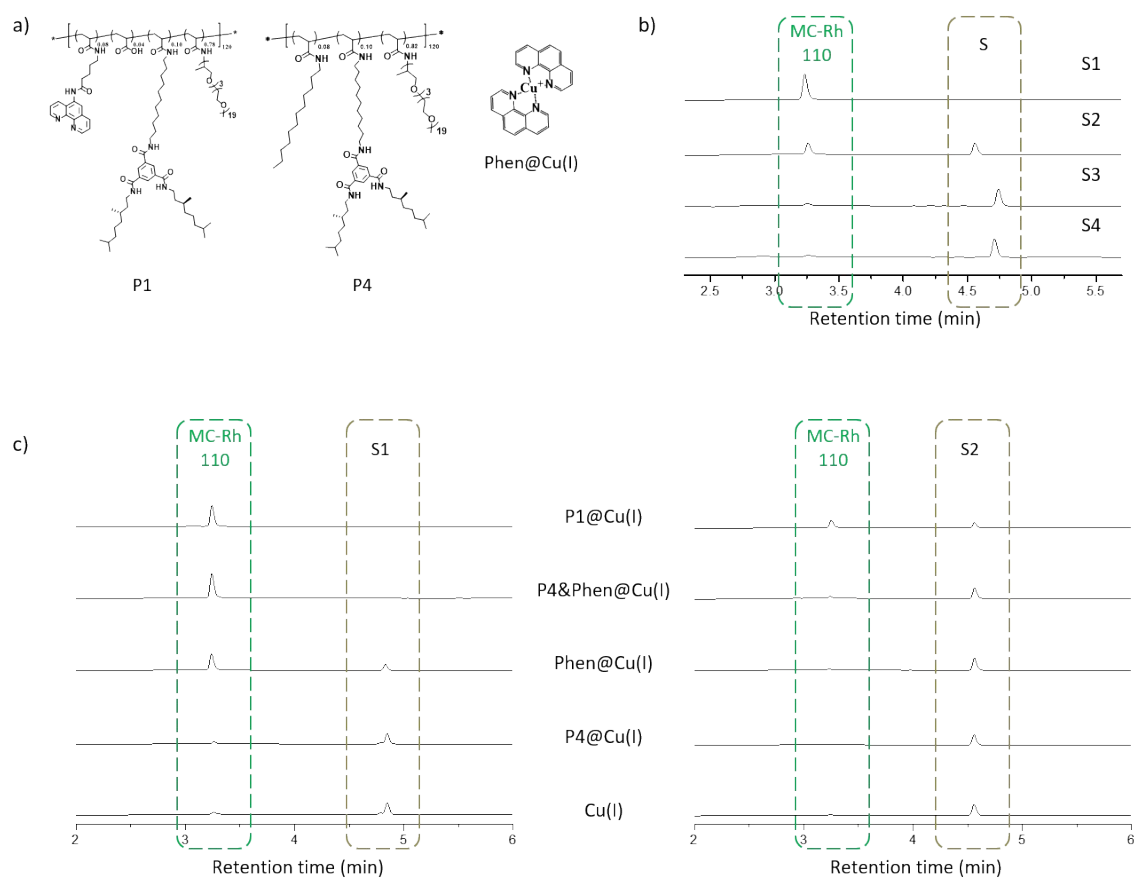
Fig. S9: a) Polymers and Phen@Cu(I); b) carbamate cleavage reaction of **S1-S4** by **P1@Cu(I)**; c) carbamate cleavage reaction of **S1** and **S2** under different catalysis conditions. (The conversions were caculated based on the integration of MC-Rh 110's absorption, **S1-P1@Cu(I)** was used as a standard of 100% conversion).
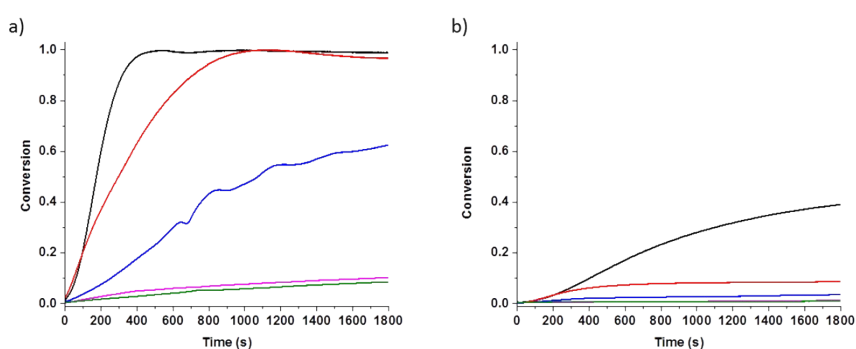
Fig. S10: Fluorescence curves (normalized by conversion) obtained from carbamate cleavage reaction of (a) **S1** and (b) **S2** in different conditions. (black: **P1@Cu(I)**; red: **P4&Phen@Cu(I)**; blue: Phen@Cu(I); pink: **P4&Cu(I)**; green: Cu(I)).

Fluorescence measurements:
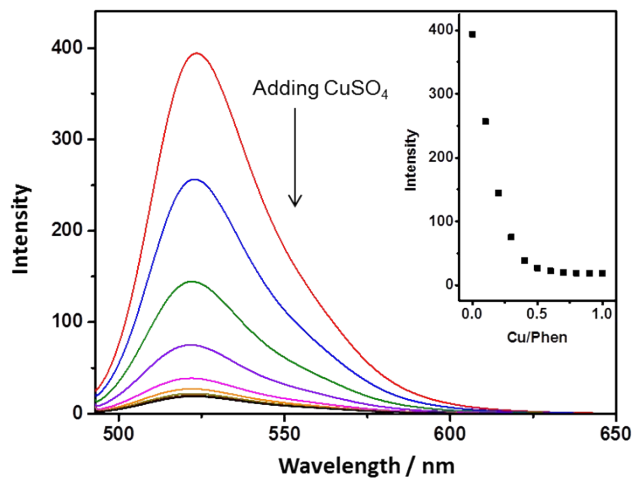
Fig. S11: Quenching of fluorescence of **P3** by addition of CuSO$_4$ ([**P3**]=10 µM).

6. Single-molecule experiments

Sample preparation for single-molecule experiments:

Cleaning of coverslips

The glass coverslips (circular, 24 mm diameter, # 1.5, Menzel Gläser) were placed in a cleaned Teflon holder, immersed in acetone (spectrophotometry grade) and sonicated for 10 min. The coverslips were then immersed in 10 % NaOH and sonicated for 10 min. Afterwards the coverslips were washed extensively with ultrapure water, followed by drying under a stream of N_2. For long term storage, the cleaned coverslips were kept in methanol.

Functionalization of coverslips

The dried, cleaned coverslips were placed into an UV-ozone cleaner for 1 h to remove any remaining impurities and to increase the number of silanol-groups on the glass surface. Subsequently, the coverslips were submerged in the freshly prepared silane solution for 30 min. The solution consisted of 2 % 3-aminopropyl dimethylethoxy silane (ABCR), 10 % ultrapure water and 88 % ethanol. After silanization, the coverslips were rinsed with ethanol to remove any unreacted silane molecules and dried under a stream of N_2.

Coupling of the amino-reactive NHS-PEG-biotin to the amino-functionalized coverslips was performed based on a published protocol.[2] Briefly, the amino-functionalized coverslips were first incubated in borate buffer (50 mM, pH 8.5) for 1 h. The coverslips were then dried under a stream of N_2. A freshly prepared solution of NHS-PEG-Biotin (50 mM in borate buffer; 50 μL) was pipetted onto one coverslip. A second coverslip was placed on top to form a sandwich. The sandwich was incubated in a water-saturated atmosphere to prevent evaporation. After 1 hour, the biotin-functionalized coverslips were rinsed with ultrapure water and dried under a stream of N_2 to remove any unreacted NHS-PEG-Biotin molecules.

The biotin-functionalized coverslips were immediately immersed in a solution containing streptavidin (150 nM in phosphate-buffered saline (PBS); 5 mL). After 1 h of continuous shaking, the samples were washed extensively with ultrapure water to remove any unbound streptavidin. Subsequently, the streptavidin-coated coverslips were immersed in a solution containing the Alexa Fluor® 488 and biotin-labeled SCPN **P3** (PBS; 5 mL). After 1 h of continuous shaking, the samples were washed extensively with PBS to remove any unbound

SCPNs. The samples were kept immersed in PBS until the measurement was started in the confocal microscope.

Confocal fluorescence microscope setup:

A custom-built confocal fluorescence microscope, optimized for single-molecule detection, was used for all single-molecule measurements. The microscope was based on an inverted optical microscope frame (Axiovert 200; Zeiss) equipped with an oil-immersion objective (Zeiss Fluar, 100x, NA = 1.3). A continuous wave solid state laser (488 nm, Genesis MX488-1000 SLM OPS Laser System, Coherent) was used for excitation. The laser light was guided to the microscope through a single-mode optical fiber (HP460, Thorlabs). An excitation filter (475/25 band-pass, Semrock) was used for cleaning up the laser light. Fluorescence emission from the sample was separated from reflections and scattered light using a dichroic mirror (505dcxr, Chroma) and a band-pass filter (525/45-25 bandpass, Semrock). The signal was then guided through a 50 μm pinhole and focused onto an avalanche photo diode detector (SPCM-AQR-14, Excelitas Technologies). The photon counts were recorded using a Picoharp 300 time-correlated single photon counting system (Picoquant). The signal was further routed into the TAO (tip-assisted optics) module of a Nanowizard I atomic force microscope (AFM; JPK Instruments) equipped with a 100×100 μm xy-scanner. The AFM software was used for scanning the confocal images. All the measurements were performed at room temperature (21 ºC). The fluorescence time traces were analysed using custom-made Matlab™ scripts.

Fluorescence Correlation Spectroscopy:

Fluorescence correlation spectroscopy (FCS) is a well-established statistical analysis method that can be used, for example, to obtain information about the size of the diffusing particles in solution.[3] In this report, a molecular diffusion model was used to obtain information about the following parameters: (1) the diffusion time and, subsequently, the hydrodynamic radius of SCPNs; (2) the concentration of product molecules in solution during catalysis. In FCS diffusion experiments, the confocal microscope is focused inside the solution containing diffusing fluorescent particles and, subsequently, a fluorescence time trace is recorded. In the next step, the time dependencies of the intensity fluctuations in the recorded time trace are analysed using an autocorrelation function. The autocorrelation function $G(\tau)$ for the measured intensity time trace $F(t)$ is defined as

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle \delta F(t) \rangle^2} \tag{1}$$

where $\delta F(t)$ and $\delta F(t + \tau)$ are the intensities at the time t and $t + \tau$, respectively. $\langle \delta F(t) \rangle$ represents the average intensity of the time trace. Physical parameters (e.g. the diffusion coefficient and/or the concentration of fluorophores) can be identified and quantified when fitting the autocorrelated data with an appropriate model. For a three-dimensional Gaussian point spread function (valid for a typical confocal detection volume) the diffusion model for one diffusing species[2] can be written as

$$G(\tau) = \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1}\left(1 + \frac{\tau}{s^2\tau_D}\right)^{-1/2} \tag{2}$$

where N is the average number of molecules in the volume, τ_D is the molecular diffusion time and s represents the structural parameter. s is defined as $s = z_0/r_0$, where z_0 and r_0 represent the $1/e^2$ radii of the vertical and horizontal direction of the confocal detection volume. The translational diffusion coefficient D is related to the molecular diffusion time as follows:

$$D = \frac{r_0^2}{4\tau_D} \tag{3}$$

Equation 3.2 can be further simplified with a two-dimensional approximation $(z_0 \rightarrow \infty)$ to a form of

$$G(\tau) = \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1} \tag{4}$$

which contains only two fit parameters. This 2D-approximation describes the data sufficiently well when using a large pinhole (50 μm), which results in a highly elliptical detection volume in the z-direction $(z_0 \gg r_0)$.

In this work, autocorrelation functions of the recorded data were calculated off-line, using a custom made Matlab™ algorithm based on photon-pair correlation.[4] The algorithm is applied directly to the photon arrival time trace and computes the correlation function from the inter-

photon distances. The main advantage of this algorithm is that it does not require any pre-processing of the data. Fitting the diffusion model to the calculated autocorrelation data was performed using custom-made Matlab™ scripts. The fitting algorithm was based on a Nelder-Mead simplex nonlinear minimization method.

Determination of the hydrodynamic radius of Alexa Fluor® 488 labeled SCPN.
FCS diffusion experiments were performed to obtain information about the size of the folded SCPNs. FCS was applied to measure the diffusion coefficient and further determine the hydrodynamic radius R_h of the Alexa Fluor 488-labeled SCPNs **P3** in water. The R_h value can be determined from Stokes-Einstein equation for freely diffusing spherical particles:

$$R_h = \frac{k_B T}{6\pi\eta D} \tag{5}$$

where k_B is the Boltzmann constant, T is the absolute temperature and η is the viscosity of the solvent. To determine R_h, the diffusion coefficient D of the sample needs to be quantified.

FCS experiments were performed using the confocal microscope setup as described earlier. To characterize the FCS setup and to quantify the horizontal radius of the confocal detection volume r_0, the fluorophore ATTO488 (1 nM, ultrapure water) was measured as a calibration standard. ATTO488 is similar in size to Alexa Fluor 488, used for labelling SCPN **P3**, hence allowing the direct comparison of the diffusion parameters. The temperature corrected reference diffusion coefficient for ATTO488, $3.59 \times 10^{-10}\, m^2 s^{-1}$ in water at room temperature, was obtained from reference.[5] In these conditions, $R_{h,ATTO488} = 0.61\, nm$.

In the FCS experiment, 100 µL of sample solution were pipetted onto the cleaned microscope coverslip. The laser focus of the microscope was positioned into the solution 5 µm above the coverslip surface. The excitation intensity was kept low (28 µW, measured in front of the excitation filter) to prevent triplet state formation. A fluorescence time trace was recorded for 50 s and the autocorrelation function (ACF) was calculated for each 10 s interval. One example of the normalized ACF of the reference dye ATTO488 and of SCPN **P3** is shown in Fig. S8 (see above). For ATTO488, each ACF was fitted with a one-component, two-dimensional

diffusion model (Eq. 4). The fits of all 10 s intervals yielded a mean diffusion time of 16.0 ± 0.6 μs, which was further used to determine $r_0 = 152 \pm 3$ nm (Eq. 3).

A visual comparison of the normalized ACFs clearly shows that the SCPN **P3** sample contains a species that diffuses significantly slower than ATTO488 in the reference sample. For SCPN **P3** (100 nM, ultrapure water), the ACFs could not be fitted with the one-component 2D-diffusion model. Instead, a two-component 2D-diffusion model was used:

$$G(\tau) = \frac{1}{N} \sum_{i=1}^{2} f_i \left(1 + \frac{\tau}{\tau_{Di}} \right)^{-1} \tag{6}$$

where f_1 and f_2 are the fractional contributions of each component.

The fits yielded diffusion times of $\tau_{D1} = 216 \pm 20$ μs and $\tau_{D2} = 6.4 \pm 1.7$ μs. The slow diffusion component τ_{D1} is approximately one order of a magnitude slower than the reference dye ATTO488. This clearly indicates that this diffusion time characterizes SCPN **P3**. Based on this diffusion time, the mean hydrodynamic radius of SCPN **P3** was determined to be $R_{h,SCPN} = 8.3 \pm 0.8$ nm (Eq. 3 and Eq. 5).

The origin of the fast component τ_{D2} is not easily explained. The fitted diffusion time τ_{D2} is approximately 2.5 times shorter than the diffusion time of the reference dye ATTO488. The decay time is also too long to originate from triplet state processes, as triplet state decay times are typically on the few microsecond time scales. A more detailed investigation would be required to fully understand the origin of this fast decay component; however, this unknown time constant does not influence the validity of the main observations. SCPN **P3** is successfully labeled with Alexa Fluor® 488 and its hydrodynamic radius is determined to be 8.3 nm, which matches with the values determined from light scattering experiments.

Monitoring the accumulation of product molecules in solution

A second series of FCS experiments was performed to determine if the surface immobilized SCPNs retain activity. In these experiments, using the substrate **S1**, the accumulation of product molecules (MC-Rh110) was monitored, following the increase in the number of

product molecules N in the detection volume. As an increase of product molecules may also originate from autohydrolysis of **S1**, the same experiment was performed using a sample that did not contain SCPN.

In the sample preparation, the streptavidin functionalized glass coverslip was fully covered with SCPN **P2**. To ensure the highest surface coverage, an excessively high concentration (6 μM) was used. First, the fluorescently labeled SCPN **P3** was used to find out if the concentration is high enough to cover the surface. For the actual FCS experiment, the SCPN without fluorescent label (**P2**) was used, to reduce the background fluorescence. The confocal scans of surfaces with immobilized SCPN with and without fluorescent label are shown in Fig. S12a. The scans clearly show that the surface was covered with SCPN **P3**. In addition, the surface that was coated with non-labeled SCPN **P2** did not show any significant fluorescence, as was expected.
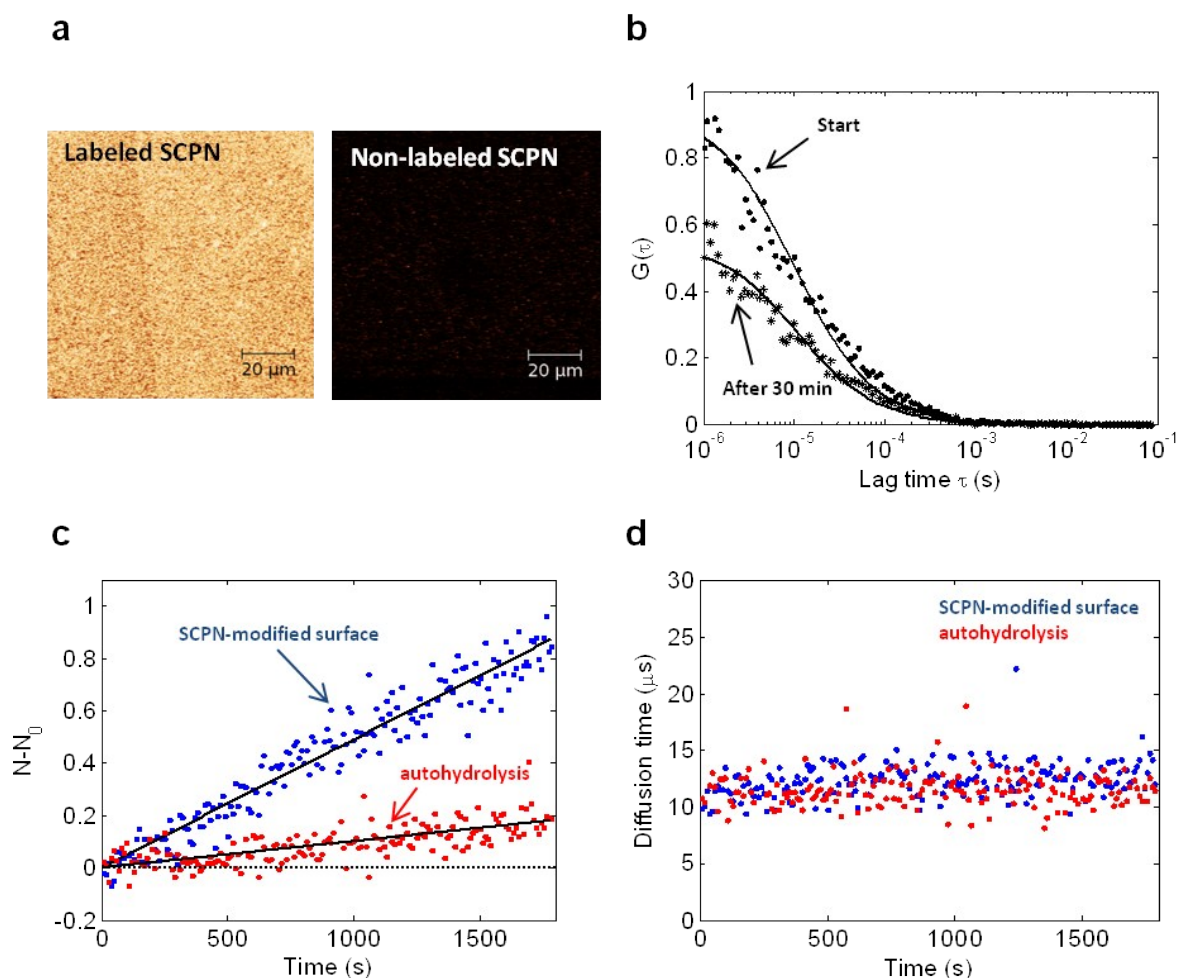
Fig. S12: FCS experiment of product accumulation. (a) Confocal images of the glass surface containing either the labeled SCPN **P3** or the non-labeled SCPN **P2**. (b) ACFs of 10 s sections taken either at the start or at the end of the measurement (after 30 min), incl. diffusion fit. (c) Time evolution of the increase of product molecules in the detection volume measured on a SCPN-modified surface (blue) and on a surface without SCPN (autohydrolysis, red) related to the number of product molecules at the start of the experiment N_0. (d) Time evolution of the diffusion times.

In the FCS experiment, 400 µL of substrate **S1** (10 µM) was pipetted onto the sample coverslip. The laser focus was positioned into the solution 5 µm above the coverslip surface. The excitation intensity was kept low (36 µW, measured in front of the excitation filter) to minimize triplet state formation and unnecessary photobleaching. A fluorescence time trace was recorded for 30 min and the autocorrelation function (ACF) was calculated for each 10 s interval (example curves are shown in Fig. S12b). The ACFs were fitted with a one-component, 2D-

diffusion fit (Eq. 4). The ACFs calculated for the first and the last 10 s interval are shown in Fig. S12b for comparison. The two ACFs illustrate the decrease of $G(0)$ (and increase of N) over time.

Fig. S12c illustrates the rate of product accumulation for the sample containing immobilized SCPN (blue) and for a control experiment with only autohydrolysis (red). The graph shows the increase in the number of product molecules related to the number of product molecules in the start of the experiment N_0. The rate of product accumulation was obtained from the slope of the fitted line. The fit yields rates of $49.1 \times 10^{-5} s^{-1}$ and $10.1 \times 10^{-5} s^{-1}$ for SCPN **P2** and autohydrolysis, respectively. The data was pre-processed before fitting, removing the clear outliers. Outliers were defined as data points where the measured value differed by more than 2 standard deviations from the local mean value (window size: ±10 datapoints).

As the observed fluorescent species remains the same during the whole measurement, no changes in diffusion time should occur during the time course of the experiment. The plotted time evolution of diffusion times Fig. S12d show that the diffusion time indeed does not change significantly.

Bleaching of the Alexa Fluor® 488 label after consecutive scanning
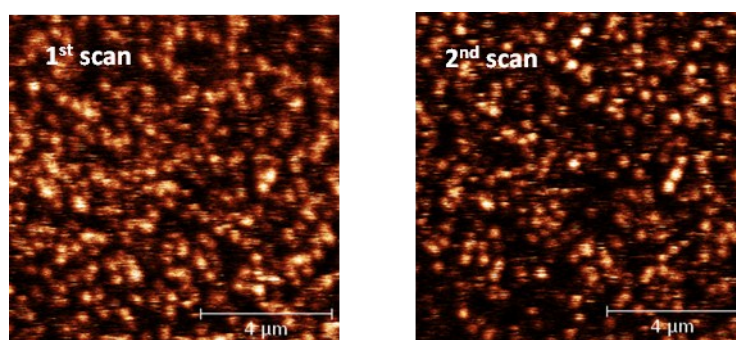
Fig. S13: Consecutive confocal images from the same area of Alexa Fluor® 488 labeled and biotin-functionalized SCPN **P3** immobilized on the streptavidin-functionalized surface and incubated in ultrapure water. The scans show that no significant fluorophore bleaching is observed with the used laser excitation intensity (10 µW).
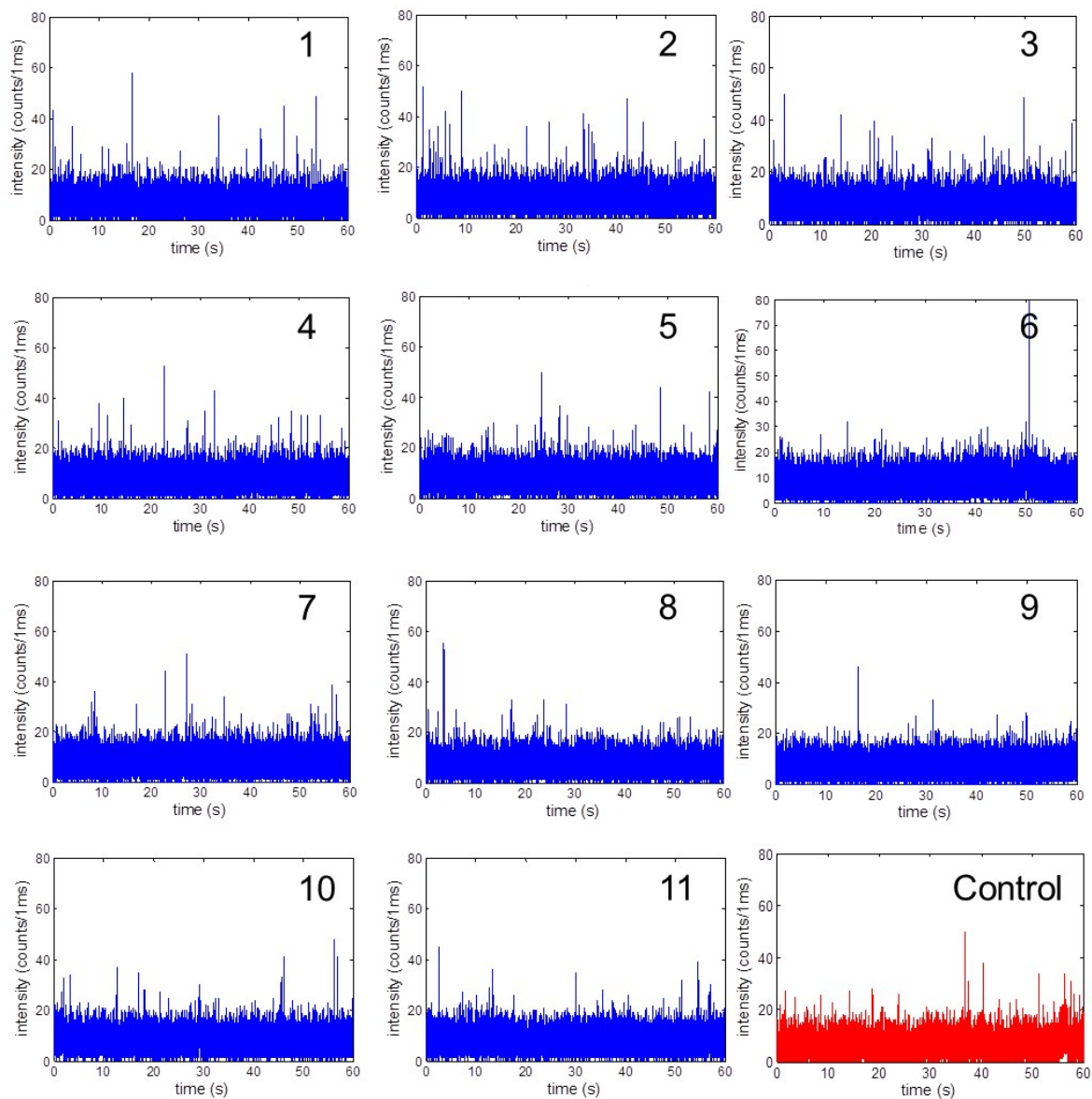
Fig. S14: Collection of representative fluorescence intensity-time traces, showing the reaction of SCPN **P3** turning over the substrate **S1**. The control time trace was measured on an "empty" area on the same surface.
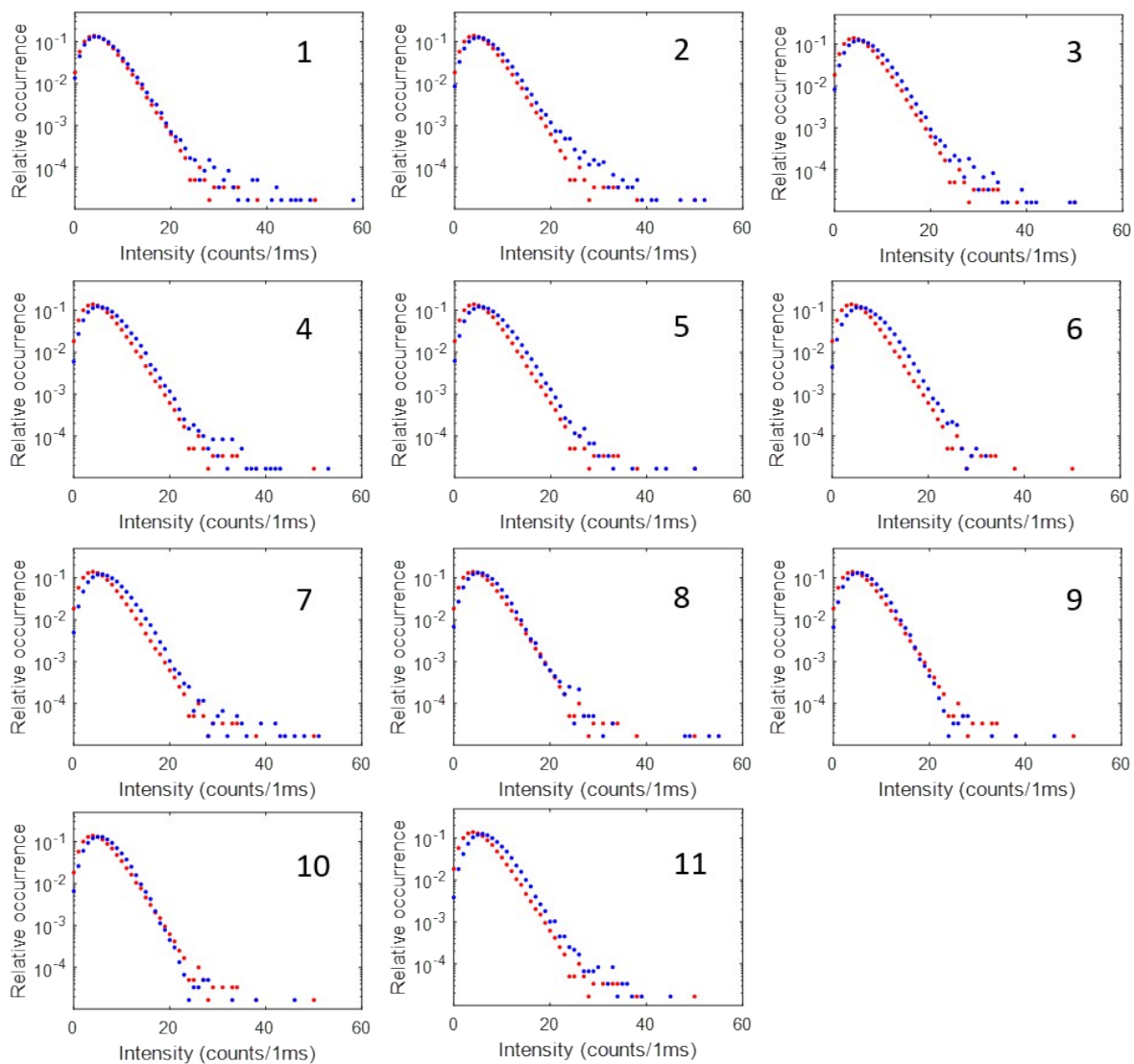
Fig. S15: Fluorescence intensity distributions of all the recorded the time traces. The data was recorded on the location of individual SCPNs (blue) and on an "empty" area on the surface (red, control). Each graph shows the data for one SCPN plotted against the control measurement for direct comparison.

7. References

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