

Electronic Supplementary Information

Fluorescent RAFT polymers bearing a nitrilotriacetic acid (NTA) ligand at the α -chain-end for the site-specific labeling of histidine-tagged proteins

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EXPERIMENTAL PART

Materials.

N-acryloyl morpholine (NAM) (Aldrich, 97%) was distilled under reduced pressure (120 °C ; 10 mmHg) to remove inhibitor. *N*-acryloxysuccinimide (NAS) was synthesized as previously described.¹ 2,2'-Azobis(isobutyronitrile) (AIBN) (Fluka, 98%) was purified by recrystallization from ethanol. 1,4-dioxane (Acros, 99%) was distilled over LiAlH₄ (110 °C). Succinimidoxycarbonyl ethyl dithiobenzoate (SEDB) was synthesized and purified according to a previously published process.² Trioxane (Acros, 99%), diisopropylethylamine (DIPEA, Sigma-Aldrich, ≥99.5%), 4-(2-Aminoethyl) morpholine (AEM, Aldrich, 99%), Dansyl cadaverine (FluoProbe), anhydrous dimethylformamide (DMF, Fisher Scientific, 99.99%), chloroform (Fisher Scientific), *N*_α-*N*_α-bis(carboxymethyl)-*L*-lysine TFA salt (NTA-NH₂, Sigma, ≥95%), nickel(II) sulfate hexahydrate (NiSO₄, Fluka, 99%) were used as received.

HisTag proteins were kindly provided by Dr. Marc Lavigne (HisTag-INT and HisTag-TOX4PG) and Pr. Philippe Bouvet (HisTag-NCL), Laboratory Joliot-Curie, ENS de Lyon, France.

Synthesis of the NTA-functionalized CTA (NTA-CTA).

SEDB (32 mg, purity >93%, 92 μmol, 1.2 eq.) and DIPEA (27 μL, 153.4 μmol) were dissolved in 750 μL of anhydrous DMF. NTA-NH₂ (29 mg, 77 μmol, 1 eq.) dissolved in 750 μL of anhydrous DMF were then added over 10 min before the addition of 20 μL of DIPEA (114.8 μmol). The mixture was placed under magnetic stirring and heated at 40°C in a thermostated oil bath for 2h. The reaction medium was then diluted in 20 mL of ethyl acetate and the mixture was extracted with 20 mL of a 10% aqueous NaCl solution. The orange aqueous phase was washed with 20 mL of ethyl acetate. It was then acidified with 2 mL of a 1M aqueous HCl solution and extracted with 20 mL of ethyl acetate. The pink organic phase was collected, washed 4 times with 20 mL of a 10% aqueous NaCl solution adjusted to pH 1 and finally dried under vacuum during 12h to give a red/pink powder (85% yield).

¹H NMR (300.13 MHz, CD₃CN): 7.97 (d, J = 7.8 Hz, 2H), 7.61 (t, J = 7.8 Hz, 1H), 7.45 (t, J = 7.8 Hz, 2H), 6.88 (t, NH), 4.59 (d, J = 7.2 Hz, 1H), 3.54 (m, 2H), 3.52 (m, 2H), 3.36 (m, J = 6 Hz, 1H), 3.15 (m, J = 6 Hz, 2H), 1.59 (d, J = 7.2 Hz, 3H), 1.25 – 1.85 (m, 6 H).

High resolution ESI-TOF mass spectrometry: *m/z* (M+Na)⁺, calculated 493.1074 ; found 493.1077

RAFT Polymerizations.

RAFT polymerization protocols were adapted from previously optimized procedures.^{3,4}

NTA-PNAM-DB homopolymers. NAM (5.67 mmol), NTA-CTA (55 μmol), AIBN (5.5 μmol) (0.06 mol.L⁻¹ in dioxane, 90.5 μL), trioxane (0.47 mmol) (internal reference for ¹H NMR determination of monomer consumption) and dioxane (2.03 mL) were introduced in a Schlenk tube equipped with a magnetic stirrer. The mixture was degassed by five freeze-evacuate-thaw cycles and then heated at 80°C under nitrogen in a thermostated oil bath. Periodically, samples were withdrawn from the polymerization medium via a canula for analyses. Individual monomer conversions are determined by ¹H NMR following the decrease of the NAM vinyl protons in comparison with the protons of trioxane.¹ After synthesis, polymers were purified by precipitation in diethyl ether and dried under vacuum.

NTA-P(NAM-stat-NAS) copolymers. Statistical copolymerization of NAM and NAS monomers followed the same procedure, starting with NAM (2.34 mmol), NAS (1.56 mmol), NTA-CTA (41 μmol), AIBN (4.1 μmol) (0.06 mol.L⁻¹ in dioxane, 67.2 μL), trioxane (0.098 mmol) and dioxane (1.59 mL).

Synthesis of the fluorescent polymer (NTA-polymer-Dansyl) and preparation of the Ni/NTA-polymer-Dansyl probe.

NTA-P(NAM-stat-NAS)-DB (30 mg, $M_n = 9\,200\text{ g.mol}^{-1}$, 3.26 μmol , 1 eq.) and Dansyl Cadaverine (2.7 mg, 8.15 μmol , 2.5 eq.) were dissolved in 1.5 mL of chloroform. DIPEA (2.8 μL , 16.3 μmol , 5 eq.) was subsequently added and the yellow solution was stirred at room temperature for 2h. AEM (49 μL , 373.7 μmol , 5 eq. per NAS units) was then added and the reaction was left another 2h under stirring at room temperature. The polymer NTA-polymer-Dansyl was finally precipitated in a large volume of diethyl ether and dried under vacuum (27.2 mg, 2.84 μmol , 96% coupling yield).

Preparation of the Ni/NTA-polymer-Dansyl probe. Two approaches were explored for the chelation of the Ni²⁺ cations. The first one consisted in mixing the NTA-polymer-Dansyl with 100 eq. of NiSO₄ in water before dialysis (Spectrum Labs, Spectra/Por 6, MWCO 2 kg.mol⁻¹), lyophilization and finally solubilization of the probe in PBS 1X buffer. The second one consisted in mixing the NTA-polymer-Dansyl with 2 eq. of NiSO₄ directly in the same PBS buffer. Both approaches led to identical results.

Analytical techniques.

¹H NMR. Spectra were recorded in deuterated chloroform (Aldrich) at 300 K on a Bruker Ultrashield spectrometer operating at 300.13 MHz.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). All mass spectra were acquired with a Voyager-DE PRO (AB Sciex, Framingham, MA) equipped with a nitrogen laser emitting at 337 nm. The instrument was operated in the linear or reflectron mode. Ions were accelerated to a final potential of 20 kV. The negative ions were detected in all cases. Mass spectra were the sum of 300 shots, and an external mass calibration was used (mixture of peptides from Sequazyme™ standards kit, AB Sciex). Samples were prepared by dissolving the polymer in THF at 10 g.L⁻¹. The matrix used for all experiments was 3-indoleacrylic acid (IAA, Sigma-Aldrich), used without further purification and dissolved in THF (0.2 M). Matrix and polymer solutions were mixed at a 9:1 v/v ratio, and then 1 μL of the mixture was deposited and dried onto the MALDI sample plate.

UV-Vis absorption and fluorescence emission. Spectra in water were acquired on a Synergy MX (BioTek) microplate reader at 20.6°C.

Dot-blot and western-blot experiments.

Low binding PVDF (PolyVinylidene DiFluoride) membranes (GE Healthcare) were first soaked in 100% Methanol, then in water and finally in a washing buffer (PBS 1X) for dot-blots or a transfer buffer (Laemmli 1X, 20% Ethanol, 0.1% SDS) for western-blots, respectively.

Dot-blot. 2 μL of each HisTag protein at different concentrations as described in the text and native streptavidin (negative control) solution were blotted on the PVDF membrane before to proceed with the blocking and the staining as described below.

Western-blot. 1 μg of each HisTag-INT protein (HisTag-INT and HisTag-TOX4PG) and 2 μg of native Streptavidin (negative control) were first resolved by a 10% SDS-PAGE gel (Sodium Do-decyl Sulfate – PolyAcrylamide Gel Electrophoresis). The usual preparation used 0.1% SDS in the stacking gel, 0.1% SDS in the separating gel, 2% SDS in the loading buffer, and 0.1% SDS in the running buffer. Samples were loaded in a mixture of 0.1% SDS, 10% glycerol, 10 mM Tris-Cl (pH 6.8), 1 mM ethylene diamine tetraacetic acid (EDTA, pH 8), and bromophenol blue (tracking dye, 0.05 mg.mL^{-1}) without pre-heating of the sample. Migration was performed on a BioRad apparatus at 20 V.cm^{-1} (constant voltage) in 25mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3 – 8.8, at room temperature until the bromophenol blue dye ran out of the gel (50–60 min). Second, the blot 'sandwich' was assembled according to the instructions provided by Biorad (Trans-Blot[®] SD Semi-Dry Transfer Cell apparatus) and transferred 1h at 20V. After transfer, the PVDF membrane was rinsed with water before to proceed with the block-ing and the staining as described below.

Blocking and staining. PVDF membranes from both dot- and western-blot experiments were blocked overnight in PBS-BSA 1% for 2h at room temperature. After washing twice with PBS-Tween20 0.05% for 1h, then with PBS 1X for 30 min, staining was performed with a PBS solution ($6.7.10^{-7}$ g.mol^{-1}) of the Ni/NTA-polymer-Dansyl probe (see above) for 2h at room temperature in the dark. The membrane was then rinsed with PBS 1X and revealed under a UV lamp at 365 nm using a Quantum ST5 apparatus (Vilber Lourmat).

NTA-CTA NMR spectrum.

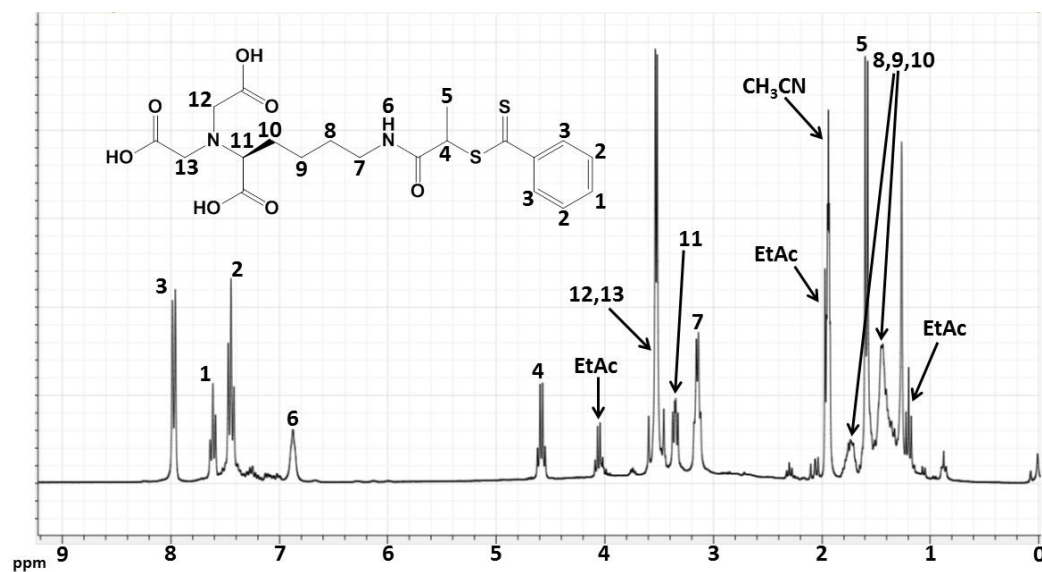
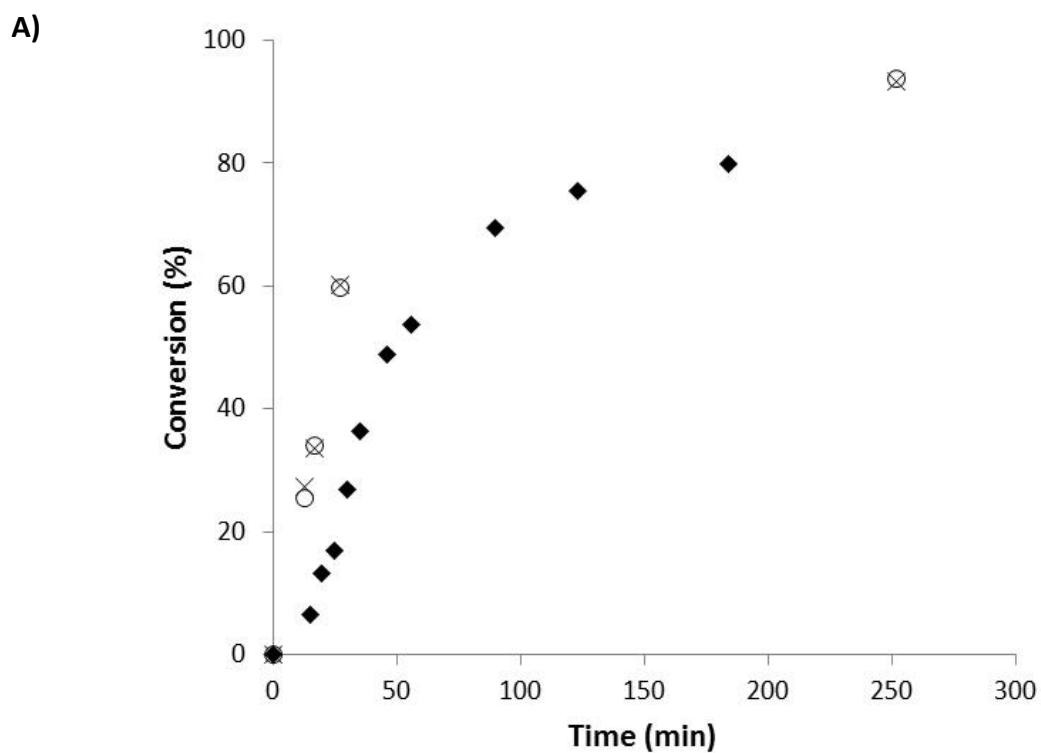


Fig. S1 ^1H NMR spectrum of the NTA-CTA (300 MHz, acetonitrile- d_3) exhibiting the expected peaks of the product (see attribution) alongside some residual ethyl acetate (EtAc) solvent and minor impurities.

RAFT NAM homopolymerization and NAM/NAS copolymerization using the NTA-CTA as control agent.



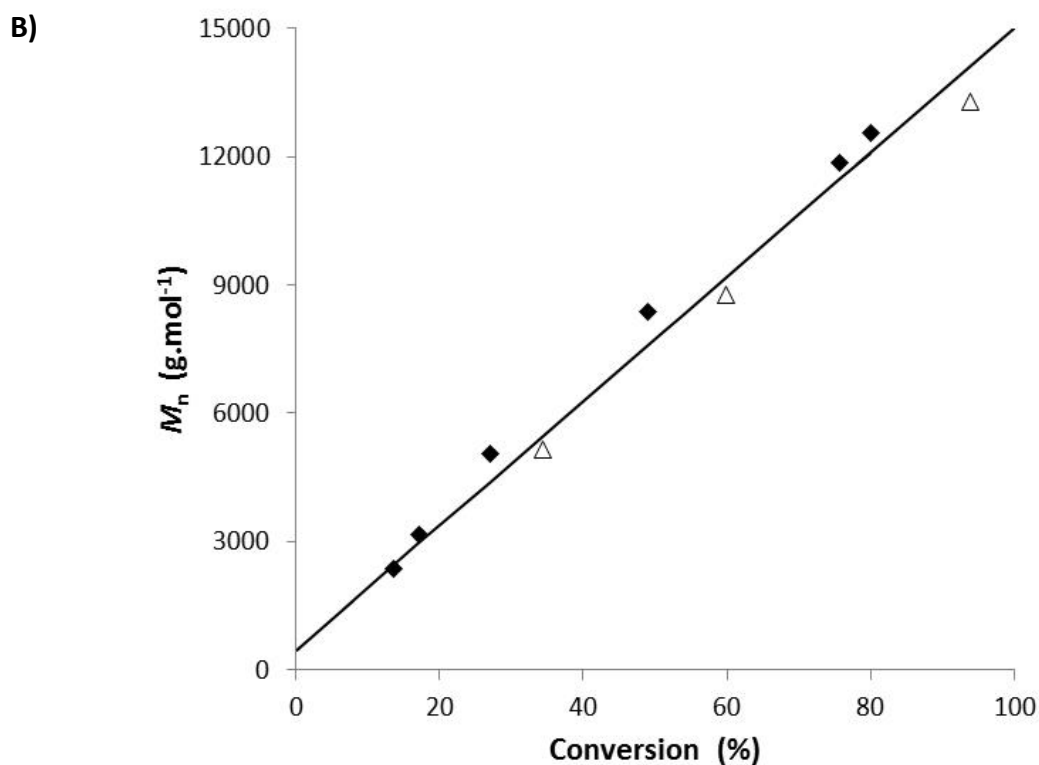
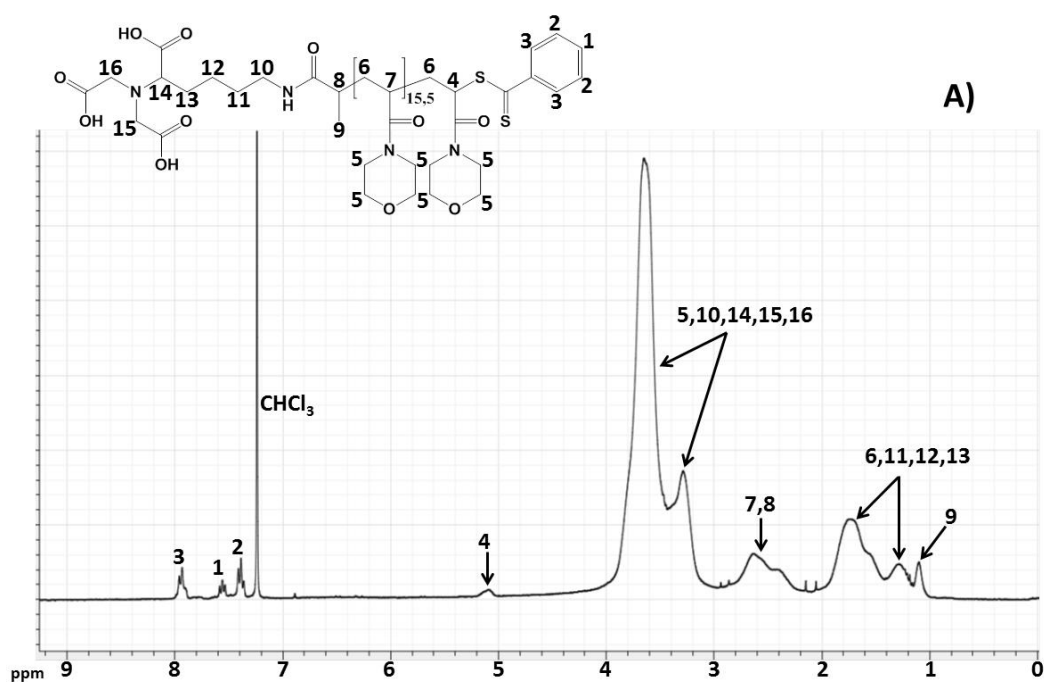


Fig. S2 A) RAFT polymerization kinetics of the NTA-CTA mediated NAM homopolymerization (diamonds) and NAM/NAS copolymerization (open circles/crosses respectively). **B)** Evolution of the number-average molecular weight M_n determined by ^1H NMR of the resulting NTA-PNAM-DB (diamonds) and NTA-P(NAM-*stat*-NAS)-DB (open triangles) polymers with conversion. Calculated M_n values (full line) were determined as previously reported.⁴



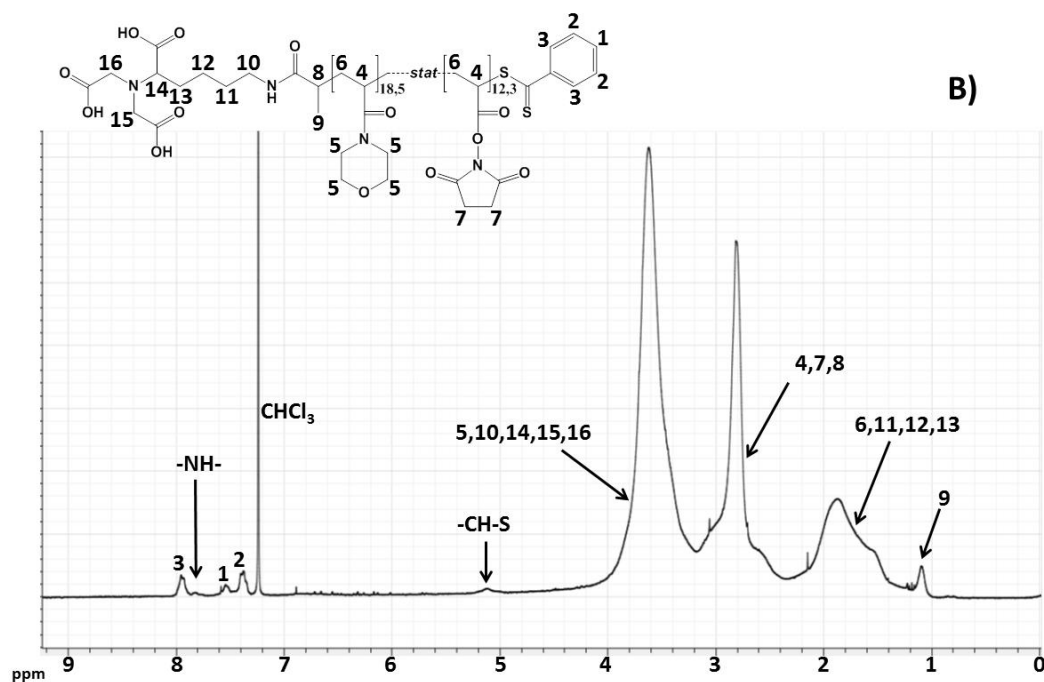


Fig. S3 ^1H NMR spectra in CDCl_3 of NTA-PNAM-DB (A) and NTA-P(NAM-*stat*-NAS)-DB (B) samples after purification by precipitation in diethyl ether.

NTA-PNAM-DB MALDI-TOF mass spectrometry.

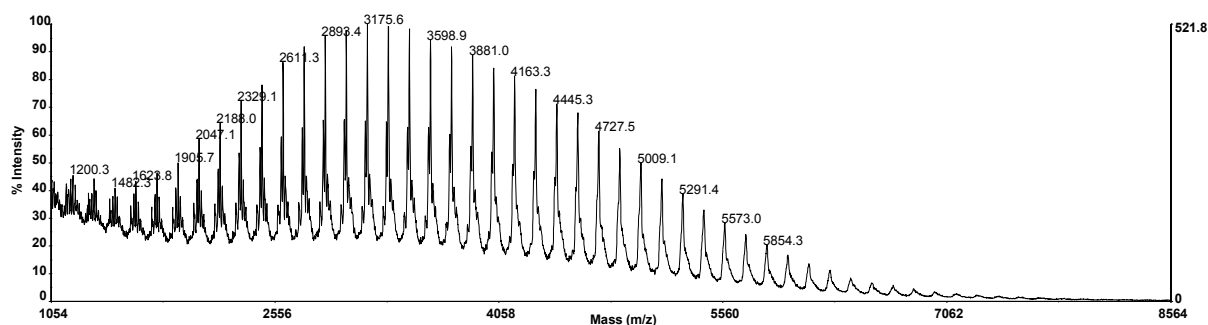


Fig. S4 MALDI-TOF mass spectrum obtained in the linear mode for a NTA-PNAM-DB sample ($M_n^{^1\text{H NMR}} = 3\,500\text{ g mol}^{-1}$). Analysis of the mass spectrum peak distribution gave $M_n = 3\,400\text{ g mol}^{-1}$ and $D = 1.18$.

Absorption and fluorescence spectra of NTA-polymer-Dansyl probe.

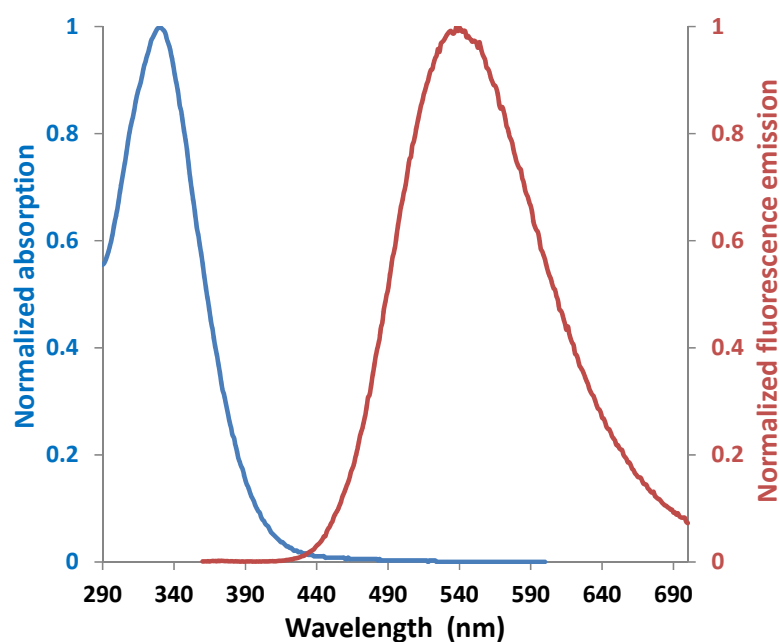


Fig. S5 Absorption (blue) and fluorescence emission (red, $\lambda_{\text{exc}}=330$ nm) spectra of NTA-polymer-Dansyl probe in water.

Absorption and fluorescence emission spectra of NTA-polymer-Dansyl probe in water were similar to the one of the free dye⁵ and showed a maximum absorption wavelength $\lambda_{\text{abs max}} = 330$ nm and a maximum fluorescence emission wavelength $\lambda_{\text{em max}} = 535$ nm.

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