Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Electronic Supplementary Information

Nanoreactors for the Multi-Functionalization of Poly-Histidine Fragments

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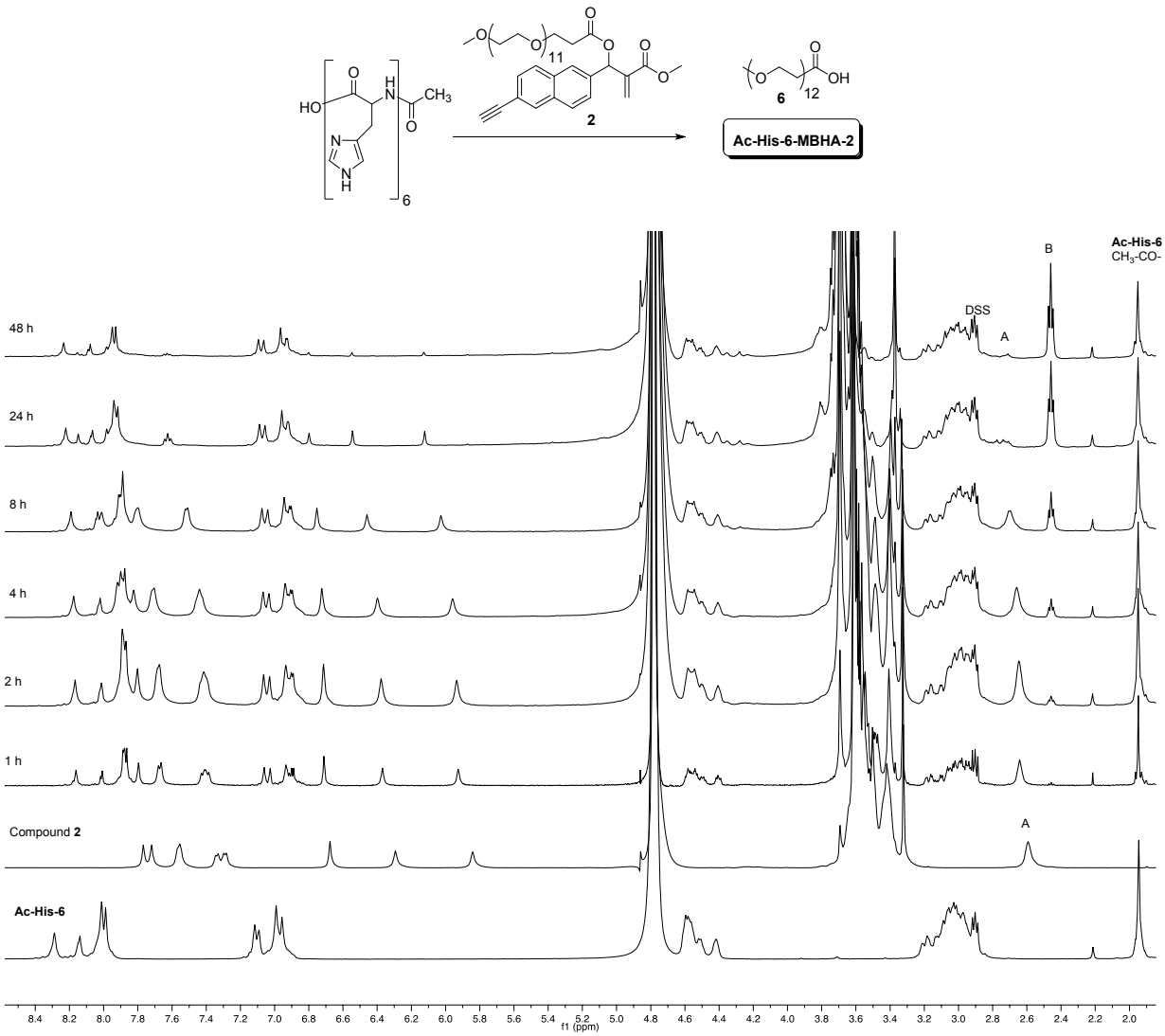

Figure ESI1. ^{1}H NMR spectra (500 MHz, PBS-D_2O) of the mixtures obtained by reaction of **Ac-His-6** with an equimolar amount of compound **2** (initial concentration 1 mg/mL). The peaks marked with A (attributed to the propionic moiety of **2**) and B (attributed to **6** produced by the concerted addition–elimination process) were used to follow the reaction progress. The peak marked with DSS was attributed to the internal standard.

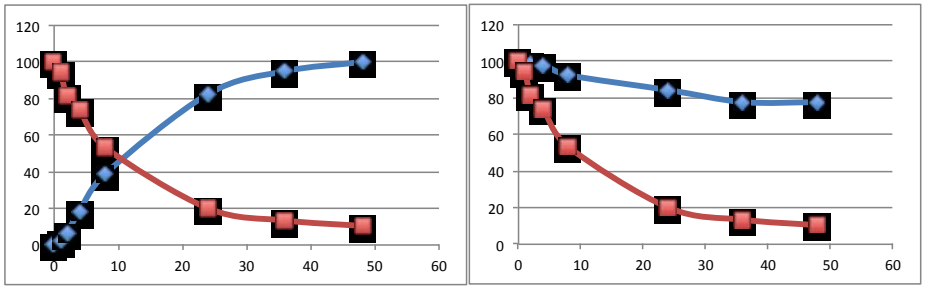

Figure ESI2. Kinetics of the reaction shown in Figure ESI1. Left panel: variation of the integral values (arbitrary units) of signal A (attributed to the propionic moiety of **2**, red) and of signal B (attributed to **6** produced by the concerted addition–elimination process, blue) in the time (h). Right panel: variation of the integral values (arbitrary units) of signal A (red) and of signal CH_3CO of **Ac-His-6** (blue) in the time (h). The residual amounts of unreacted **Ac-His-6** could be estimated with some uncertainness due to the presence of monosubstituted species, which could contribute to the integral value of signal CH_3CO of **Ac-His-6**.

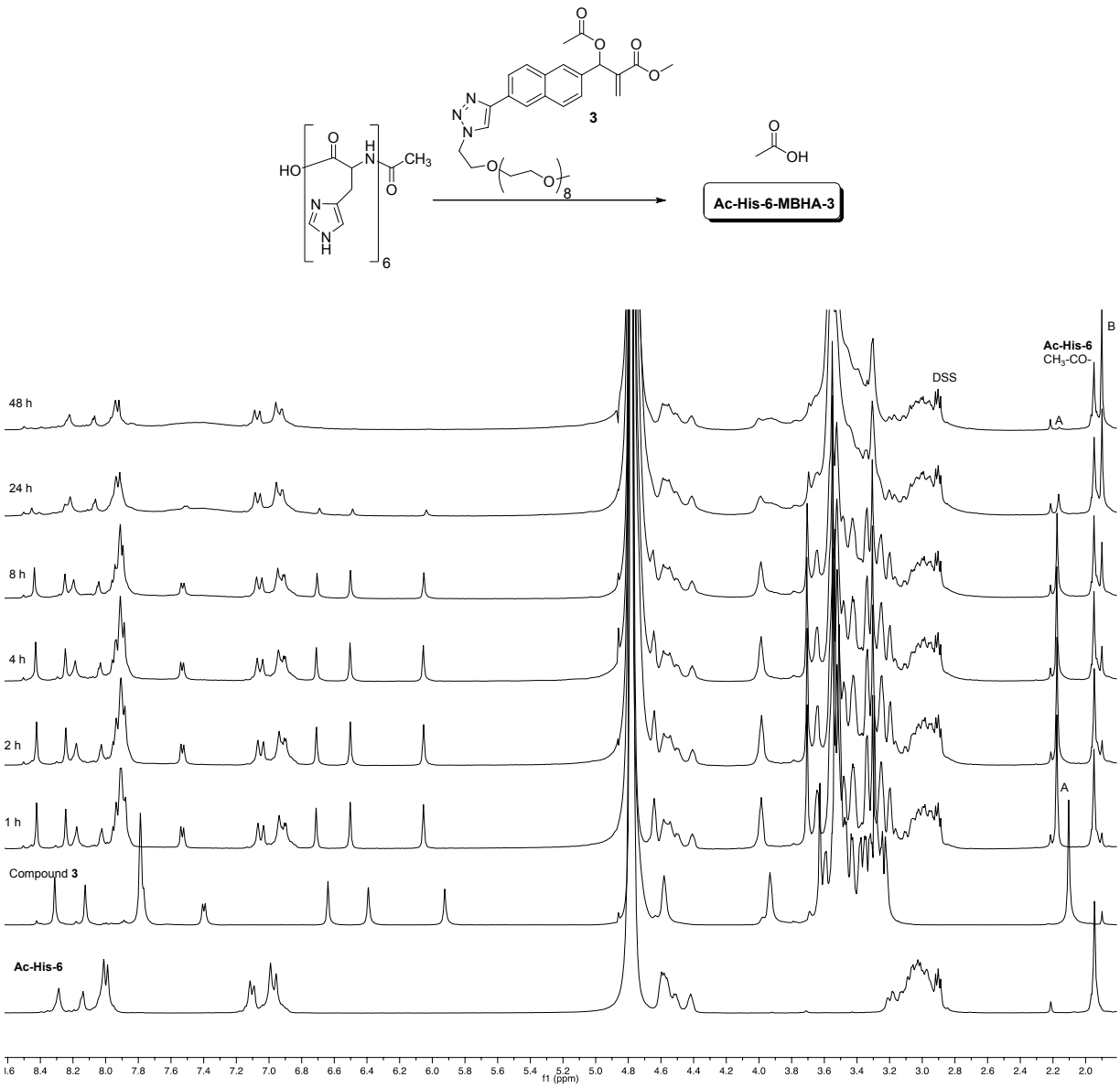

Figure ESI3. ^{1}H NMR spectra (500 MHz, PBS-D_2O) of the mixtures obtained by reaction of **Ac-His-6** with an equimolar amount of compound **3** (initial concentration 1 mg/mL). The peaks marked with A (attributed to the acetyl moiety of **3**) and B (attributed to the acetate produced by the concerted addition–elimination process) were used to follow the reaction progress. The peak marked with DSS was attributed to the internal standard.

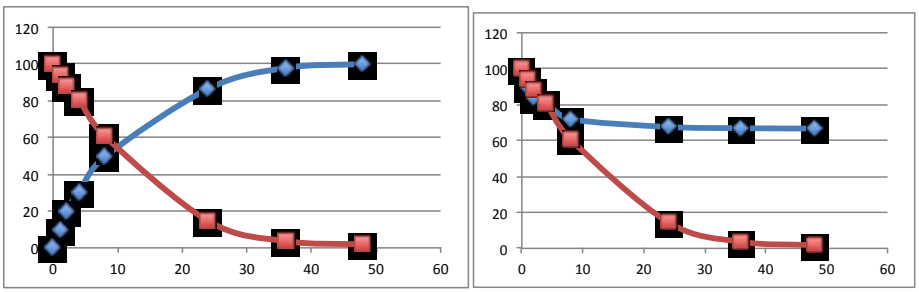

Figure ESI4. Kinetics of the reaction shown in Figure ESI3. Left panel: variation of the integral values (arbitrary units) of signal A (attributed to the acetyl moiety of **3**, red) and of signal B (attributed to the acetate produced by the concerted addition–elimination process) in the time (h). Right panel: variation of the integral values (arbitrary units) of signal A (red) and of signal CH_3CO

of **Ac-His-6** (blue) in the time (h). The residual amounts of unreacted **Ac-His-6** could be estimated with some uncertainness due to the presence of monosubstituted species, which could contribute to the integral value of signal CH_3CO of **Ac-His-6**.

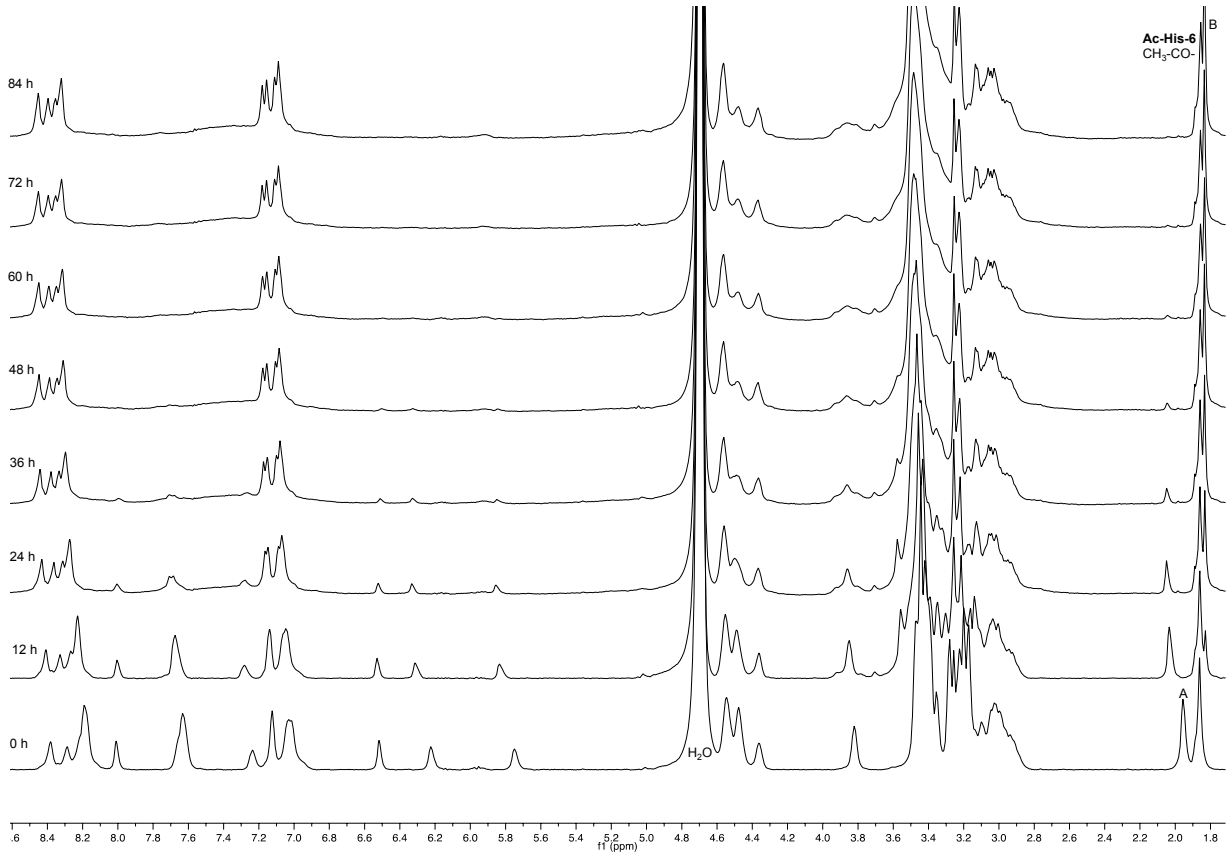

Figure ESI5. ^1H NMR spectra (500 MHz, PBS-D_2O) of the mixtures obtained by reaction of **Ac-His-6** with an equimolar amount of compound **3** (initial concentration 3 mg/mL). The peaks marked with A (attributed to the acetyl moiety of **3**) and B (attributed to acetic acid produced by the concerted addition–elimination process) were used to follow the reaction progress.

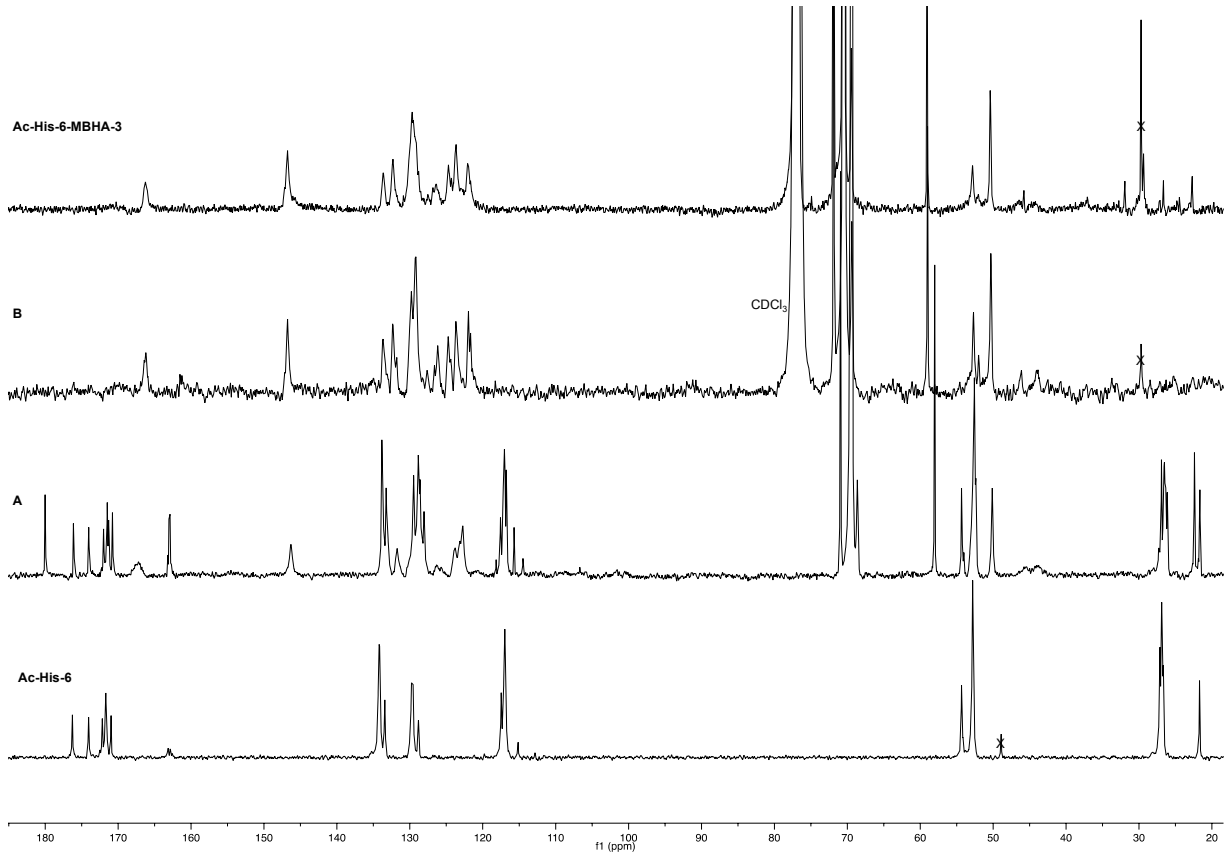

Figure ESI6. Comparison of ^{13}C NMR spectrum (237.5 MHz, PBS-D_2O) of the mixture obtained by reaction of **Ac-His-6** with an equimolar amount of compound **3** (initial concentration 5 mg/mL) after 3 weeks at room temperature (**A**) with that (125 MHz, $CDCl_3$) of the polymeric substance extracted from the reaction mixture with $CDCl_3$ (**B**), with that (125 MHz, PBS-D_2O) of starting **Ac-His-6**, and with that (125 MHz, $CDCl_3$) of previously published[1] polymeric material **Ac-His-6-MBHA-3** obtained by reaction of **Ac-His-6** with a ten-fold excess of **3**.

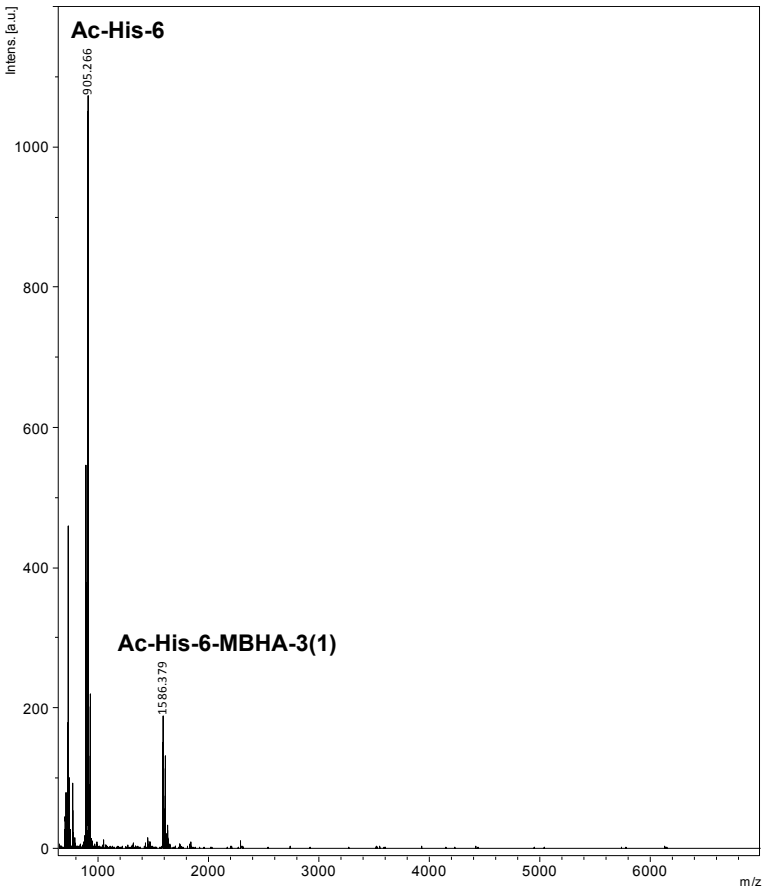

Figure ESI7. MALDI-TOF mass spectrum (positive-ion mode) of the PBS solution obtained after the extraction with chloroform of the reaction mixture of **Ac-His-6** with equimolar amounts of **3**. The peak marked with Ac-His-6 was assigned to the sodiated molecular ion of **Ac-His-6** the one marked with Ac-His-6-MBHA-3(1) was assigned to the protonated molecular ion of the reaction product bearing one naphthalene substituent.

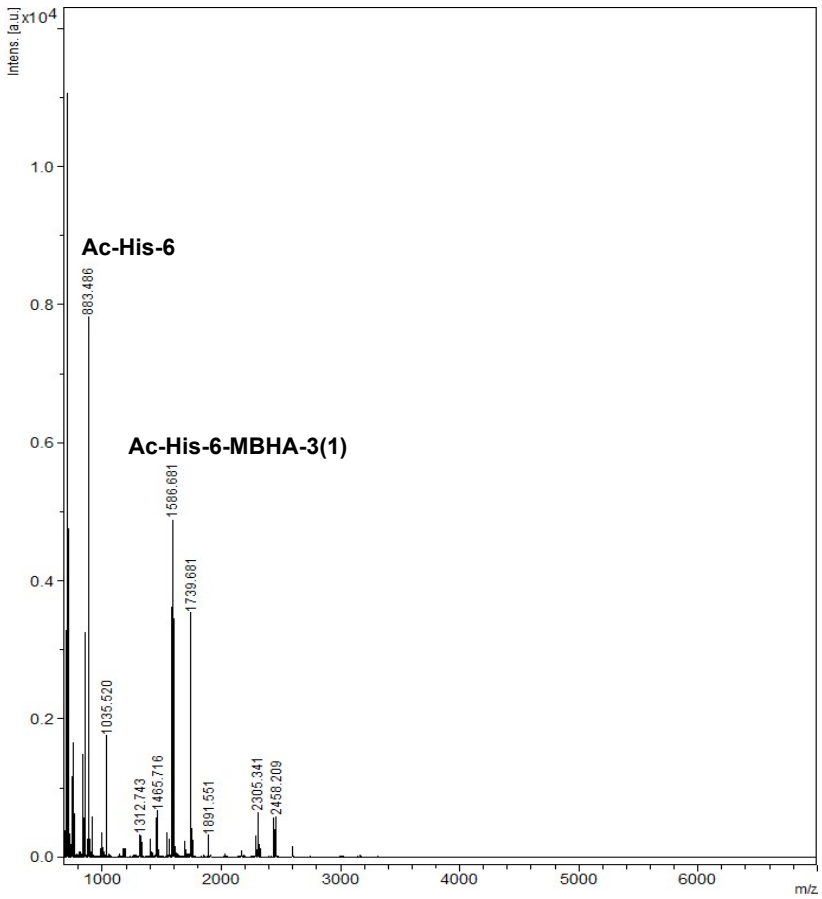

Figure ESI8. MALDI-TOF mass spectrum (positive-ion mode) of the edges of the spot obtained with the reaction mixture of **Ac-His-6** with equimolar amounts of **3**. The peak marked with Ac-His-6 was assigned to the protonated molecular ion of **Ac-His-6,** while the one marked with Ac-His-6-MBHA-3(1) was assigned to the protonated molecular ion of the reaction product bearing one naphthalene substituent.

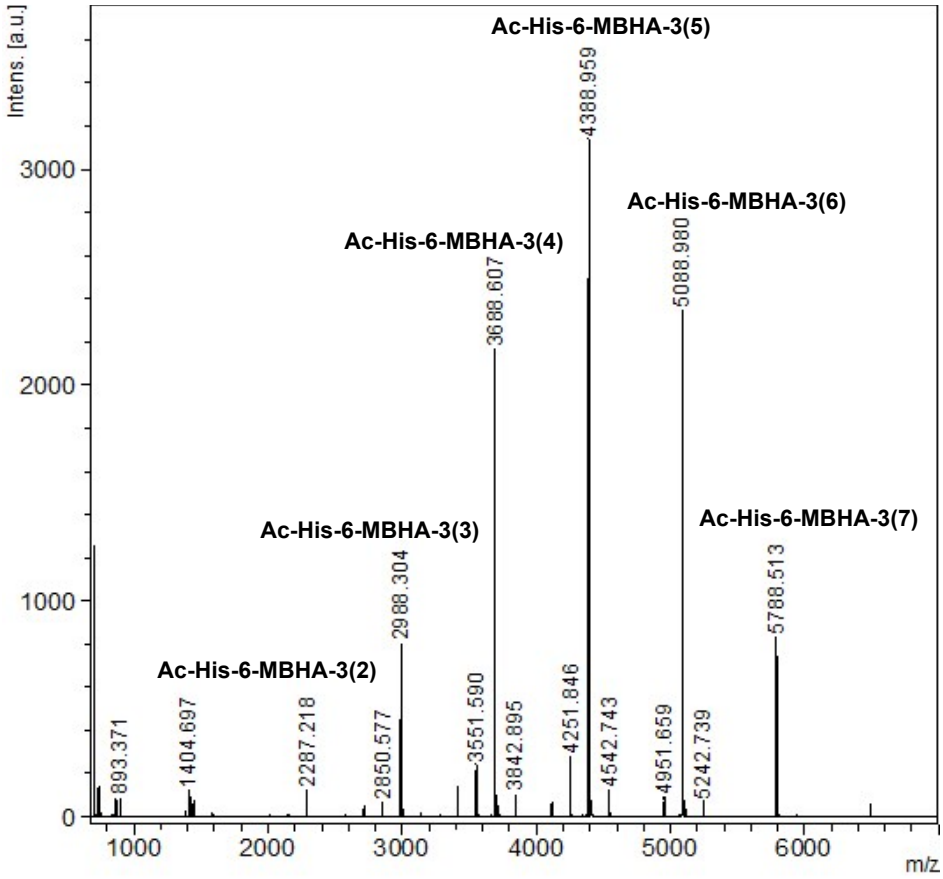

Figure ESI9. MALDI-TOF mass spectrum (positive-ion mode) of the center of the spot obtained with the reaction mixture of **Ac-His-6** with equimolar amounts of **3**. In each assignment, the value in parentheses is the number of naphthalene substituents (i. e. the grafting degree) for each **Ac-His-6** molecule.

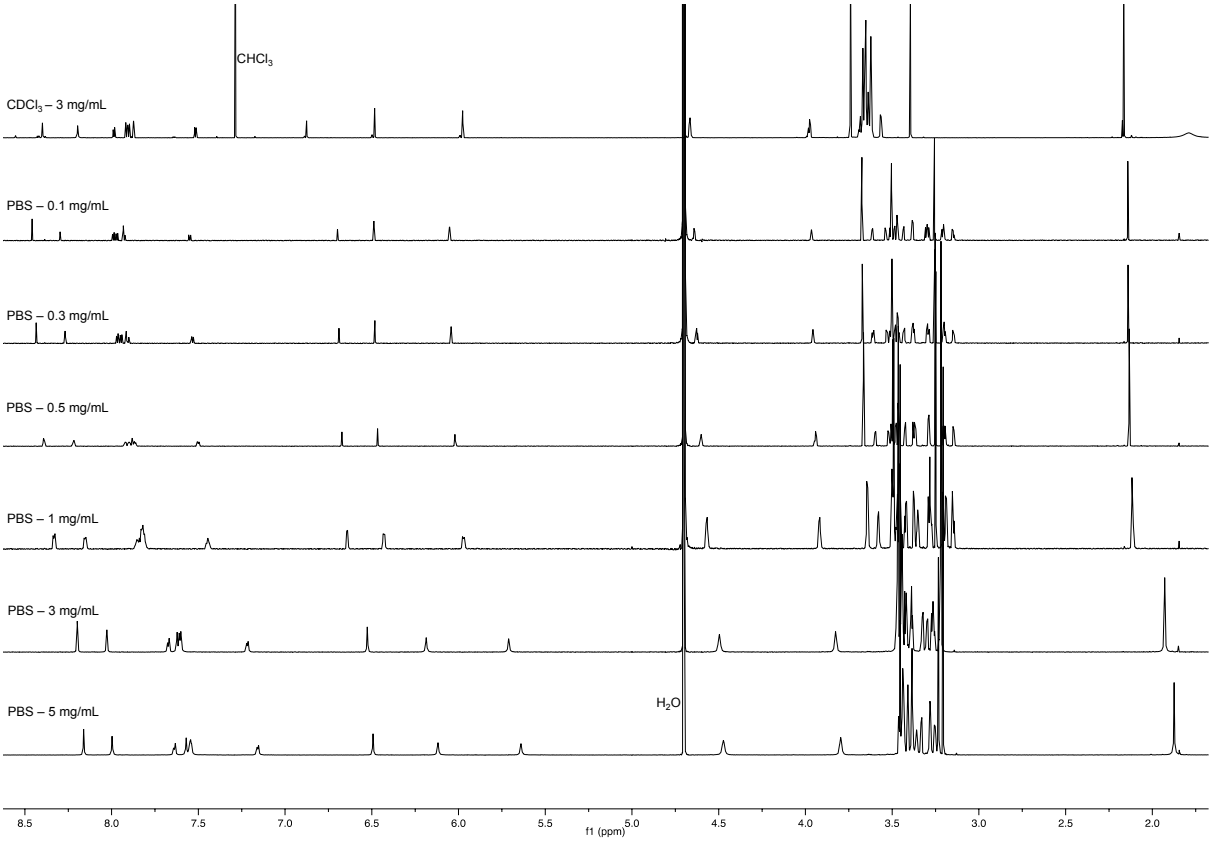

Figure ESI10. Comparison of the ^{1}H NMR spectra (950 MHz) obtained with **3** solutions in PBS showing increasing concentrations with that obtained in $CDCl_3$.

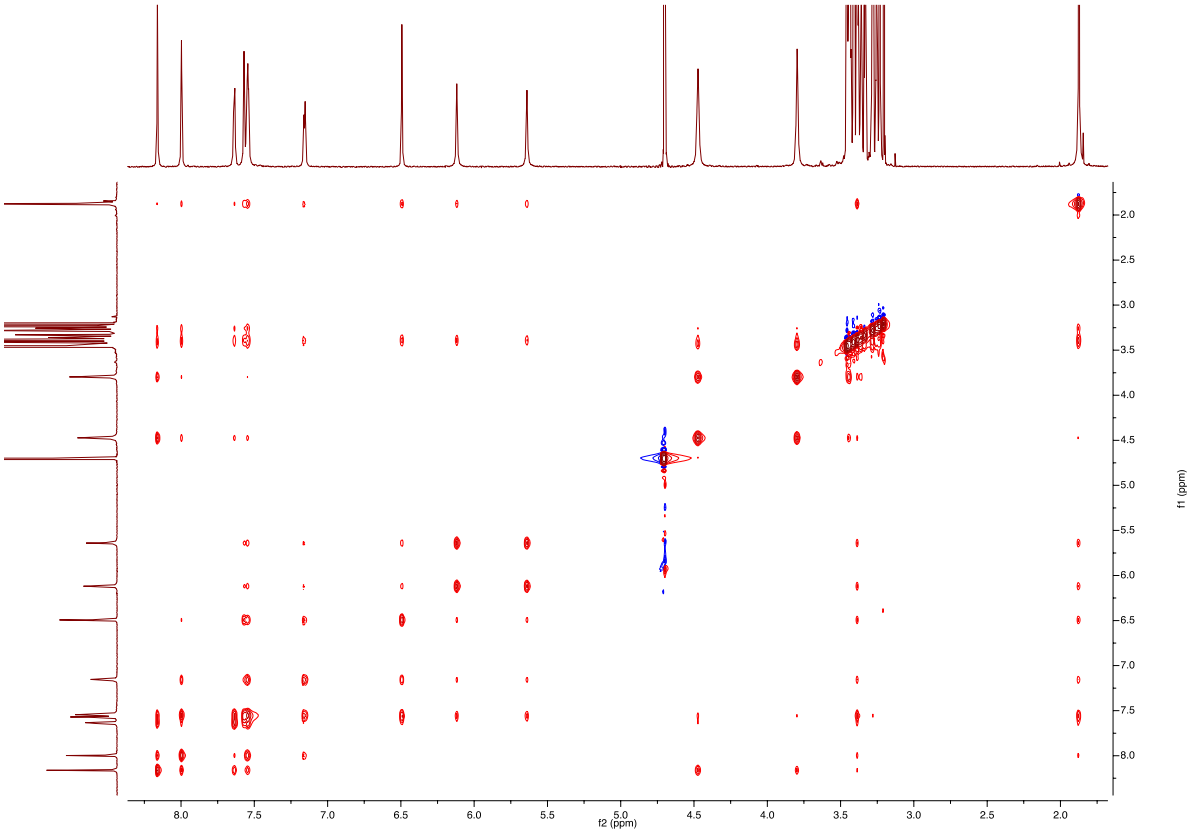

Figure ESI11. NOESY spectra (950 MHz) obtained with **3** in PBS-D_2O (5 mg/mL).

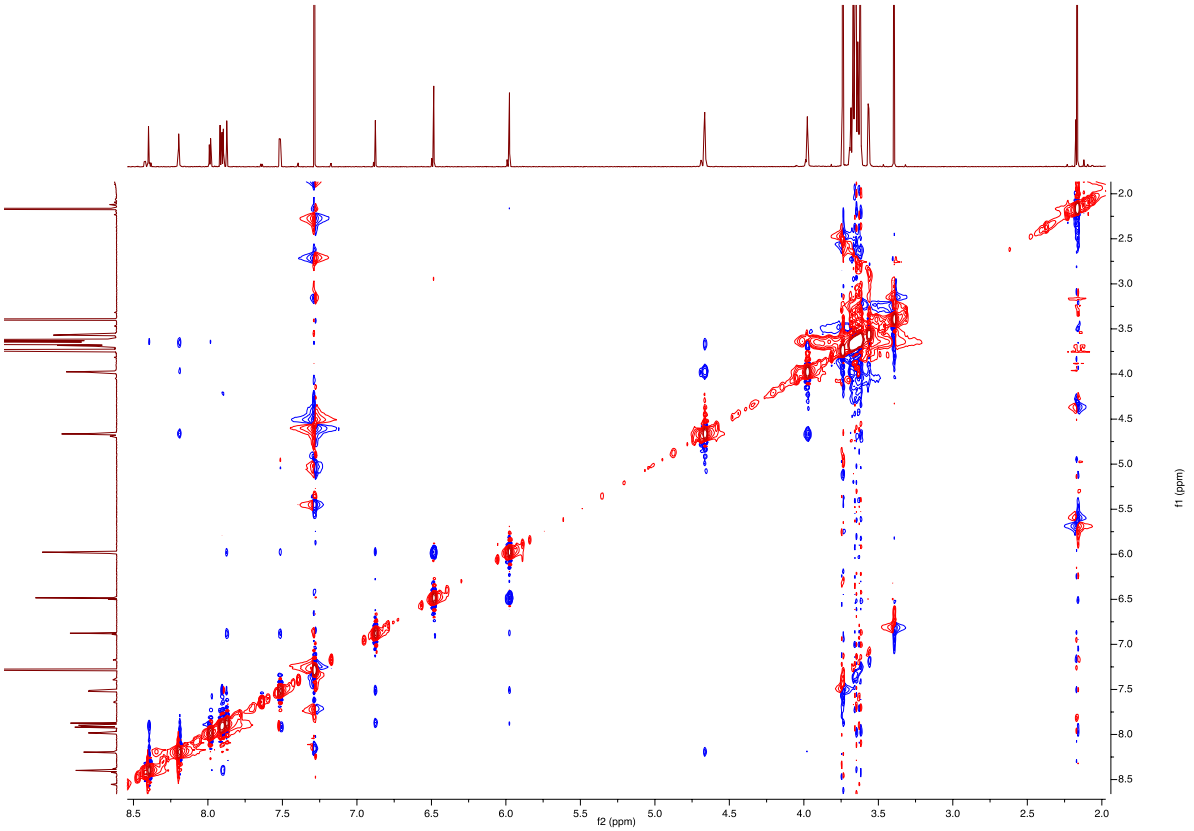

Figure ESI12. NOESY spectra (950 MHz) obtained with **3** in $CDCl_3$ (3 mg/mL).

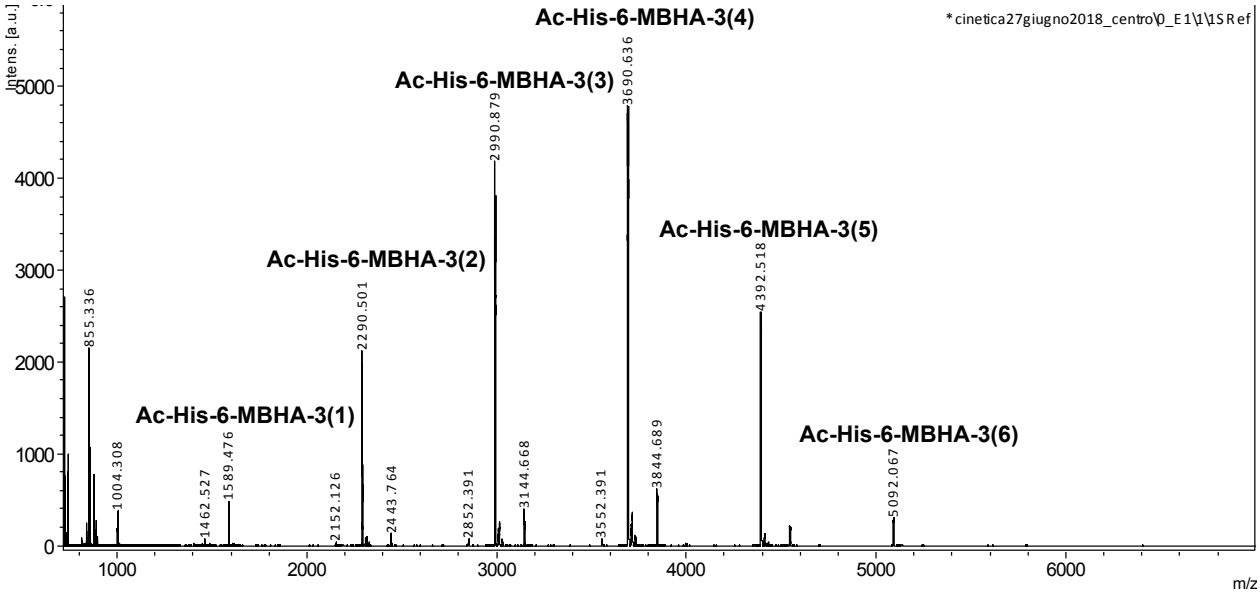

Figure ESI13. MALDI-TOF mass spectrum (positive-ion mode) of the center of the spot obtained with the reaction mixture of **Ac-His-6** with equimolar amounts of **3** at a concentration (i. e. 0.5 mg/mL) below the CAC (i. e. 0.68 mg/mL). In each assignment, the value in parentheses is the number of naphthalene substituents (i. e. the grafting degree) for each **Ac-His-6** molecule.

Table ESI1. DLS results obtained with dispersions of MBHA **2** and **3** in PBS at 25 °C.

Sample	*Za1* (nm)	PDI
2	167	0.32
3	204	0.27

Table ESI2. Energy (kJ/mol) for each **Ac-His-6-MBHA-3** conjugate.

Biadduct substitution	Energy (kJ/mol) $*10^5$	Biadduct substitution	Energy (kJ/mol) $*10^5$
1 2 3	-1.107	2 3 4	-1.297
1 2 4	-1.219	2 3 5	-1.248
1 2 5	-1.279	2 3 6	-1.175
1 2 6	-1.270	2 4 5	-1.232
1 3 4	-1.087	2 4 6	-1.063
1 3 5	-1.146	2 5 6	-1.314
1 3 6	-1.014	3 4 5	-1.138
1 4 5	-1.299	3 4 6	-1.104
1 4 6	-1.105	3 5 6	-1.303
1 5 6	-1.397	4 5 6	-1.168

Experimental section

Determination of CAC values by fluorescence analysis. A stock solution of pyrene (6.0×10^{-5} M in acetone) was prepared and then aliquots of 5 μL were placed into vials and evaporated to remove acetone in an orbital shaker at 37 °C. Subsequently, 0.5 mL of the MBHA derivative solution in PBS at pH 7.4, at concentrations ranging from 1×10^{-5} to 10 mg/mL were added to the pyrene residue; the final concentration of pyrene was 6.0×10^{-7} M in each sample. The solutions were kept at 37 °C for 24 h under continuous stirring to equilibrate pyrene with micelles. Pyrene emission spectra were recorded at 37 °C using an excitation wavelength of 333 nm. The CAC of the compounds was calculated by plotting the I_{373}/I_{384} (I_1/I_3) ratio, obtained from the emission spectra recorded at 37 °C, versus the logarithm of the polymer concentration.

DLS measurements. DLS measurements were performed at 25 °C using a Malvern Zetasizer NanoZS instrument, fitted with a 532 nm laser at a fixed scattering angle of 173° with dispersions of **2** and **3** in PBS.

Cryo transmission electron microscopy (CryoTEM) experiments.

Vitrified specimens were prepared by adding 2.5 μL to freshly plasma cleaned QUANTIFOIL® R 2/1 300-mesh copper grids. A Vitrobot Mark IV (FEI) was used to plunge freeze the grids in liquid ethane after the application of 2.5 μL MBHA derivative **3** solution (4.5 mg/mL) with 100% humidity at 20 °C, a blot time of 3 s, the blot force set to -3, and a wait time of 2 s.

The MBHA images were collected on CM 200 FEG (Philps) operating at 200 kV, equipped with a TVIPS F224HD CCD camera using TVIPS EM-Menu software for image acquisition.

NMR spectroscopy studies. NMR spectra were recorded by means of either a Bruker 950 AVANCE NEO or a Bruker DRX 500 AVANCE spectrometer both equipped with cryoprobes in the indicated solvents.

Kinetic experiments by NMR spectroscopy. The suitable compounds were accurately weighted in glass vials and dissolved in deuterium oxide (i. e. 1.0 mL) containing phosphate buffered saline (PBS, pH 7.4) powder (Aldrich, i. e. 10 mg). The solutions of the reactants were mixed in glass vials and then transferred in 5 mm NMR tubes; the reaction progress was monitored by performing ^{1}H NMR experiments at regular time intervals.[1]

Mass spectrometry measurements. MALDI–TOF mass spectra were recorded in reflectron mode by means of a Bruker Ultraflex III MALDI-TOF/TOF instrument equipped with a Nd:YAG laser at a wavelength of 355 nm with <500 ps pulse and 200 Hz firing rate. The accelerating voltage was 22 kV. External calibration was performed using a Bruker calibration mixture consisting of polypeptides with different molar mass values. All measurements were performed in positive ion mode. Approximately 2000 laser shots were accumulated for each mass spectrum, and 2,5-dihydroxybenzoic acid was used as the matrix in the analysis of all the samples.

Molecular Dynamics Simulations. The structure of N-acetylhexahistidine was built using the

Avogadro software,[2] with a deprotonated C-terminal. On the basis of the experimental observations, three histidine residues in the hexahistidine peptide were replaced by MBHA-**3** biadduct residues, according to the crystallographic structure of the previous published biadduct compound reported in reference 1. The **Ac-His-6-MBHA-3** conjugates obtained are named according to the number of the histidine carrying the MBHA-**3** moieties, as shown in Figure 4(a).

The simulation files were built using the Antachamber package[3] and converted in the Gromacs format[4] using the Acpype tool. The United-Atom Force Field Amber ff03UA[5] were used. The TIP3P model[6] was used for the water molecules. Periodic boundary conditions with a triclinic box were applyed. A distance of 1.0 nm from the **Ac-His-6-MBHA-3** conjugate surfaces to the box walls was ensured. A number of positive and negative ions, Na^+ and Cl^- respectively, were added in order to simulate an aqueous 150 mM electrolyte solution and neutralize the system. Electrostatic interactions were calculated using the particle-mesh Ewald summation method (PME) with a cutoff of 1.0 nm.[7,8] The temperature of simulations was set to 310 K and kept constant using a velocity-rescaling thermostat with a constant of 0.1 ps. The pressure was set to 1 bar to mimic biological conditions and it was controlled by a Parrinello-Rhaman barostat with a constant of 2.0 ps and an isothermal compressibility of $4.5 \bullet 10^{-5}$, characteristic of water.

Each **Ac-His-6-MBHA-3** conjugate was first equilibrated for 2 ns in the NVT ensemble and, successively, for 2 ns in the NPT ensemble. After the equilibration steps, the production run for data analysis were performed for 100 ns. Molecular dynamics simulations and analysis were performed with the Gromacs 5.0.4 software.[4]

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