Aqueous synthesis and self-assembly of bioactive and thermo-responsive HA-*b*-ELP bioconjugates

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EXPERIMENTAL SECTION

Materials

Starting biomacromolecules: Sodium hyaluronate (HA 5K, 20K, and 40K) were purchased from Lifecore Biomedical. For each size, the same batch was used for all the experiments, with precise molar masses of 4.6 kD, 24 kD, and 42 kD respectively. ELP[M_1V_3-n] with n = 60, 80, and 100 (*i.e.*, ELPn60, ELPn80 and ELPn100) were produced following previously reported procedures.[1]

Solvents: Ethanol (96.0%, EtOH), methanol (98.5%, MeOH), dimethylformamide (DMF, 99%), acetonitrile (99.9%, ACN), diethyl ether, and tetrahydrofuran (THF) were purchased from VWR and used as received. D_2O was provided by Eurisotop. Ultrapure water (18 MΩ-cm) was obtained by using a Millipore Milli-Q Biocel A10 purification unit.

Reactants: Acetic acid (AcOH, 99.8%), sodium cyanoborohyde (NaBH3CN, 95%), 3-Azido-1-propanamine (90%), sinapic acid, and Trizma® were purchased from Sigma-Aldrich. *N,N* Diisopropylethylamine (DIPEA,99%), sodium L- (AcONa, 99%), trifluoroacetic acid (TFA) , sodium nitrate (NaNO3) were acquired from Alfa Aesar. Ammonium acetate and sodium hydroxide (NaOH) were purchased from Fisher Scientific (FR), sodium hydrogenophosphate (Na_2HPO_4) from VWR, and DBCO-C6-NHS ester (DBCO) from Lumiprobe.

Synthesis

Saline buffers preparation

Saline TRIS buffer (TRISs) was prepared with 0.05 M Trisma-HCl (Trizma) and 0.15 M NaCl in ultrapure water. pH was adjusted to 7.4 with a 0.1M NaOH solution.

[1] Dai, M.; Georgilis, E.; Goudounet, G.; Garbay, B.; Pille, J.; van Hest, J.C.M.; Schultze, X.; Garanger, E.; Lecommandoux, S. Refining the Design of Diblock Elastin-Like Polypeptides for Self-Assembly into Nanoparticles. *Polymers* **2021**,*13*,1470. https:// doi.org/10.3390/polym13091470

PBS buffer was prepared by dilution of a 10X PBS commercial solution (*Euromedex*) at pH 7.0. Salinity was adjusted at 300 mosm with concentrated PBS and pH increased at 7.4 with a 0.1M NaOH solution.

Synthesis of hyaluronan-azide (HA-N_3)

Hyaluronan $HA_{4.6k}$ (500 mg, 110 µmol, 1 eq.) was dissolved in 3 mL of acetate buffer (AcONa/AcOH, 2M, pH 5,5) at 50°C. After addition of *N*-(3-azidopropyl)-*O*-methylhydroxylamine (390 mg, 3 mmol, 30 eq) and sodium cyanoborohydride (62.84 mg, 1 mmol, 10 eq), the solution was let under stirring at 50°C for 5 days (Thermomixer, 600 rpm). It was then purified by dialysis (1 kD pores membrane). The first 2 baths were done against a mixture of H2O/EtOH (1:1), and the next 4 against ultrapure water. After freeze-drying, the final product is a brittle white solid. Mass yield $r_{HA4.6k}$ = 84% (N=2).

A similar procedure has been followed for the functionalization of HA_{24k} and HA_{42k}, except for the use of a 3-Azido-1-propanamine as azide linker (commercially available) and smaller quantities: 100 mg of HA, and 20 equivalents of 3-Azido-1-propanamine and $NaBH_3CN$. Mean mass yield are respectively r_{HA24k} = 86% (N=3) and r_{HA42k} = 82% (N=5)

Synthesis of ELP-DBCO

ELPn80 (200 mg, 5.93 µmol, 1 eq.) was dissolved in 2 mL DMF under inert atmosphere (Ar). DIPEA was added (3.07 mg, 23.74 µmol, 4 eq.), followed by DBCO-C6-NHS ester (12.8 mg, 29.67 µmol, 5 eq.). The reactive medium was let stirring under inert atmosphere and shielded from light at room temperature for 2 days. Purification was carried out by precipitation in cold diethyl ether. ELP was then retrieved in the pellet after centrifugation at low temperature and dissolved again in cold ultrapure water. The solution was dialysed (3.5 kD pores membrane) against ultrapure water during 1-4 days (6 baths). Final product obtained after freeze-drying was a white fluffy solid. Mean mass yield r_{ELPn80} = 85% (N=8). A similar procedure was applied to the functionalization of ELPn60 and ELPn100 with respective mean mass yields of r_{ELPn60} = 87% (N=2) and $r_{ELPn100}$ = 91% (N=5).

Synthesis of HA-ELP bioconjugates (SPAAC)

DBCO-ELPn80 (20 mg, 0.59 µmol, 1 eq.) and $HA_{4.6k}$-N_3 (1.37 mg, 0.29 µmol, 0.5 eq.) were separately dissolved in 500 µL of ultrapure water, and mixed together. The 1 mL solution was then put under inert atmosphere (argon) and let stirring at 4°C for 2 days. The purification was achieved by centrifugation at high temperature: 5 min heating of the solution with a ThermomixerTM Eppendorf (*ThermoFisher Scientific*) followed by a 5 min centrifugation at 35°C and 10,000g. The supernatant containing the HA-ELP bioconjugates was then separated from the pellet containing the DBCO-ELP in excess. After freeze-drying, the final product is a white fluffy solid. The mean mass yield is $r_{HA4.6k\text{-}ELPn80}$ = 95% and the mean reaction yield η = 78% (N=12). The same protocol was applied to the synthesis of all the 9 bioconjugates of the library. The mean reaction yields are displayed in the Table below.

Mass yield = total final mass (bioconjugates + DBCO in excess) / initial reactant mass x 100

Reaction yield = dry supernatant mass (bioconjugates only) / theoretical mass (if 100% conversion) x 100

Table S1: Mean reaction yields for the synthesis of the HA-ELP bioconjugates library by SPAAC

Reaction yield (%)	HA		
ELP	HA 4.6k	HA 24k	HA 42k
ELP n60	**84%** ± 19 (N=4)	**74%** ± 17 (N=4)	**95%** ± 3 (N=3)
ELP n80	**78%** ± 10 (N=12)	**62%** ± 11 (N=6)	**71%** ± 13 (N=7)
ELP n100	**80%** ± 12 (N=6)	**55%** ± 6 (N=5)	**78%** ± 12 (N=5)

Recycling:

The DBCO-ELPn80 in excess present in the pellet was re-dissolved in 1 mL of ultrapure water and freeze dried. It was then used again in a new SPAAC reaction following the protocol described above. For $HA_{4.6k}$-ELPn80 synthesis with recycled DBCO-ELPn80 the mass yield was $r_{HA4.6k\text{-}ELPn80}$ = 91% and the reaction yield η = 39% (N=2).

Characterization

Gel electrophoresis

Electrophoresis analysis were carried on SDS-PAGE gels (*BioRad MiniPROTEAN TGX, non-stained, gradient 4%-20%*), on samples taken in the medium before purification (b.p.) and after centrifugation at 35°C for 5 min in the supernatant (sup) and the pellet (pel). The samples were prepared by dilution in deionized water by a ratio 1/5 (b.p.), 3/10 (sup) and 1/10 (pel), and addition of Laemmli buffer (*Biorad*). The electrophoresis was carried on a volume of 10 µL in each lane, in a Tris/Glycine/SDS buffer (*Biorad*), with a BioRad MiniPROTEAN® Tetra System device. The tension was kept constant at 300 mV and the intensity has been fixed at 25 mA per gel for 45 min. After rinse the gel was imaged with a BioRad GelDoc™ EZ Imager.

Nuclear Magnetic resonance analysis (NMR)

^{1}H NMR, ^{13}C NMR, HSQC and COSY spectra were acquired at 277K (4°C) on a Bruker Advance NEO 400 spectrometer equipped with a cryo-probe (multinuclear z-gradient direct cryoprobe-head, 5mm, *Bruker*), and at 298K (25°C) on a Bruker AVANCE III HD-400 spectrometer. Both work at 400.3 MHz.

Samples were prepared from freeze-dried compounds by their dissolution at 8 mg/mL in 400 µL of D_2O *(Eurisotop)*. The solvent peak was selected as reference (δ = 4.69 ppm at 277 K) and the spectra processed on TopSpin software.

Size exclusion chromatography

The SEC analysis were performed on an Ultimate 3000 device (*Thermoscientific*®) equipped with triple detection: it includes a diode array detector (DAD), a multi-angles light scattering detector (MALS, 18 angles), and a differential refractive index detector (dRI) from Wyatt technology. The eluent was a phosphate buffer (0.1M $NaNO_3$; 0.05M HPO_4^{2-}; pH 9). The samples were prepared in this buffer at a concentration of 2 mg/mL, with ethylene glycol as flow marker. The compounds were separated on Shodex OH Pack KD columns: a first SB G (guard 6*40) followed by two SB 804 HQ (8 * 300) with size exclusion of 5 x 10^3 – 4 x 10^5 g/mol. Measurements were performed at a flowrate of 0.6 mL/min at a pressure of 50 bar and columns temperature was held at 26°C.

For the ELPs alone, the eluent was a mixture of acetonitrile (ACN, 35%) and aqueous buffer (65%) of acetic acid (0.3M) and ammonium acetate (0.2M). The samples were prepared in this buffer at a concentration of 2 mg/mL, with ethylene glycol as flow marker. The separation was done on two TOSOH columns (7.8 * 300) with respective size exclusion < 10^6 g/mol and < 2 x 10^5 g/mol.

Data were processed on Astra software and molar masses were calculated with the dn/dC value of 0.1391 previously measured for ELPn40.

Mass spectrometry (MALDI)

Mass scpetrometry analysis were performed at the CESAMO analysis platform of the Institute of Molecular Sciences (ISM, UMR 5255). They were done by matrix-assisted laser desorption/ionization (MALDI) on a MALDI-TOF (*Autoflex maX TOF, Brucker Daltonics*) spectrometer equipped with a SMART-bean II (Nd:YAG, 355 nm) laser.

The samples were prepared by dissolution of the freeze-dried compounds in a mixture H_2O/ACN (1:1) at a concentration of 10 mg/mL. The matrix is a solution of sinapic acid at 10 mg/mL in H_2O/ACN (1:1) with TFA 0.1%. The final solution was obtained by mixing the sample solution with the matrix in a 1:9 volume ratio. A drop of 1.5 µL was deposited on an analysis plate and let to dry. The deposit and analysis were done in duplicate. Measurements were done in positive linear mode without calibration. A measurement of the compound before functionalization is systematically done to serve as reference.

Dynamic Light Scattering Measurements (DLS)

Dynamic light scattering measurements were performed on NanoZS instrument (*Malvern*, λ = 633 nm) at a 173° angle at a constant position in the cuvette (constant scattering volume). Quartz cuvettes were used (*Hellma Analytics High Precision Cell with Quartz SUPRASIL,* optical path 3x3 mm) for a volume of 70 µL. The solutions of bioconjugates were prepared in PBS (300 mosm, pH 7.4) at 78 µM. Precise z-average values and size distributions were recorded at temperature below (4-5°C) or above

(40-50°C) CMT. The automatic mode recorded 3 measurements of 13-16 runs of 10s. The evolutions of derived count rate (DCR) and z-average with temperature were plotted during a heating ramp or cycles of heating-cooling ramps. 3 measurements of 3 runs of 10s were recorded every 2°C.

Stability of the nanoparticles over time was assessed by DLS measurement of a solution of $HA_{4.6k}$-ELPn80 at 78 µM in PBS (300 mosm, pH 7.4) kept at 37°C. One measurement of 13-16 runs of 10s was done every 1h for 14h.

Zeta potential measurements

Zeta potential measurements were done on a Zetasizer Ultra device (Malvern Panalytical) in corresponding cells (Zetasizer Nano Series, DTS1070, Malvern) using the diffusion barrier method. A solution of $HA_{4.6k}$-ELPn80 bioconjugate was prepared at 26 µM in an HEPES buffer (20 mM). The analysis cell was filled with 1 mL of this same buffer. A volume of 100 µL of sample solution was then slowly injected at the bottom of the cell and 3 measurement of 3 sec. performed at 60°C. The first period (0-1.25 sec.) correspond to the Fast Field Reversal (FFR) mode and the second one (1.25-3 sec.) to the Slow Field Reversal (SFR) mode giving access to the mean value of zeta potential and distribution, respectively.

Transmission Electron Microscopy (TEM)

The TEM imaging was performed at the Bordeaux Imaging Center (BIC) on a Hitachi H7650 microscope (80 kV) equipped with a SC1000 ORIUS 11 Mpx (*GATAN*) camera.

The sample of nanoparticles of $HA_{4,6k}$-ELPn80 was prepared at 76 µM (1 mg/mL) in ultra-ultrapure water. The solution was heated at 70°C for 5 min and 5 µL were dropped on a carbon grid at 80°C and let to dry for 1 min. The stain was done by the addition of 5 µL of samarium acetate (*Sigma Aldrich*) in solution at 20 mg/mL heated at 70°C and let to dry for 1 min.

SUPPLEMENTARY FIGURES

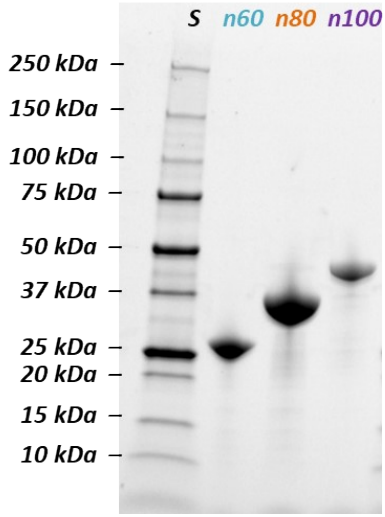

Figure S1: *SDS-PAGE analysis of the recombinant ELP[M_1V_3-n] (n = 60, 80, 100). S: Protein size markers.*

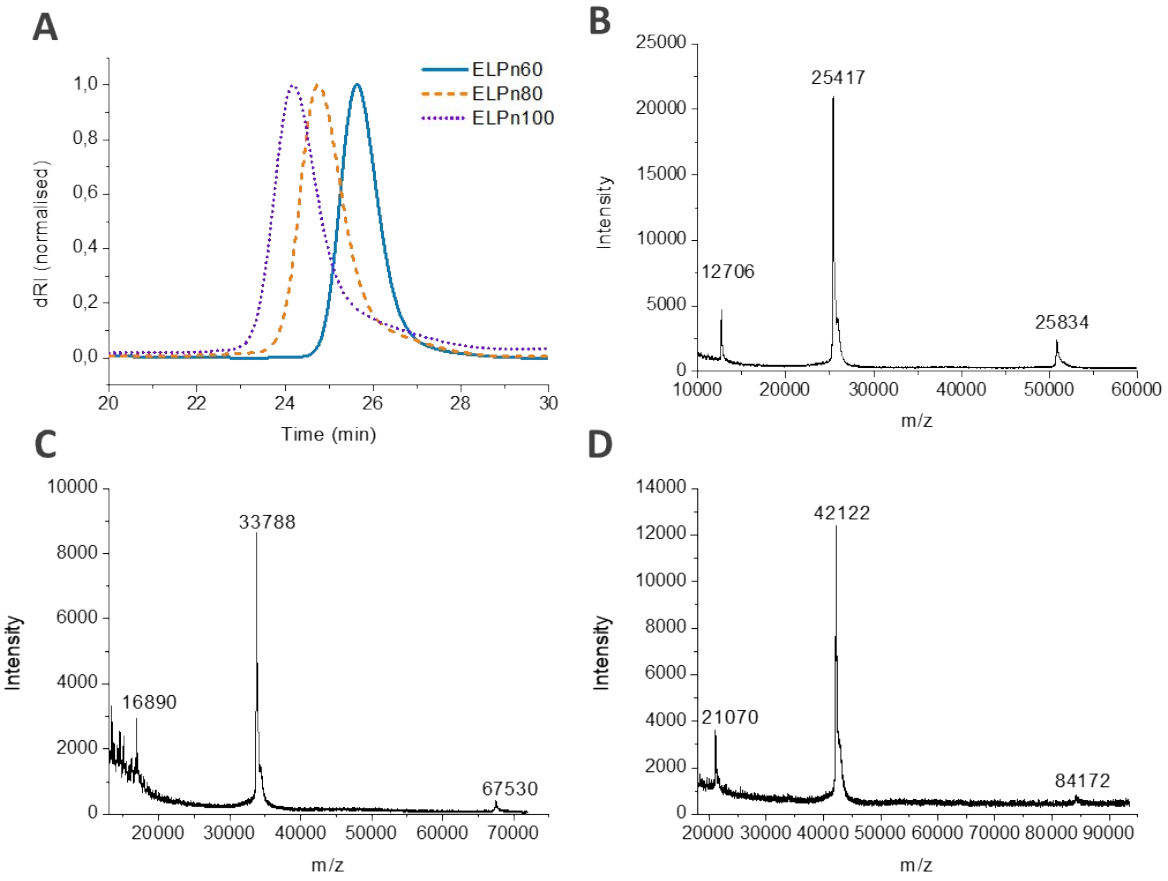

Figure S2: *(A) Size exclusion chromatograms of the ELP[M_1V_3-n] (n = 60, 80, 100), ammonium acetate buffer; (B-D) MALDI mass spectra of ELPn60 (B), ELPn80 (C), ELPn100 (D).*

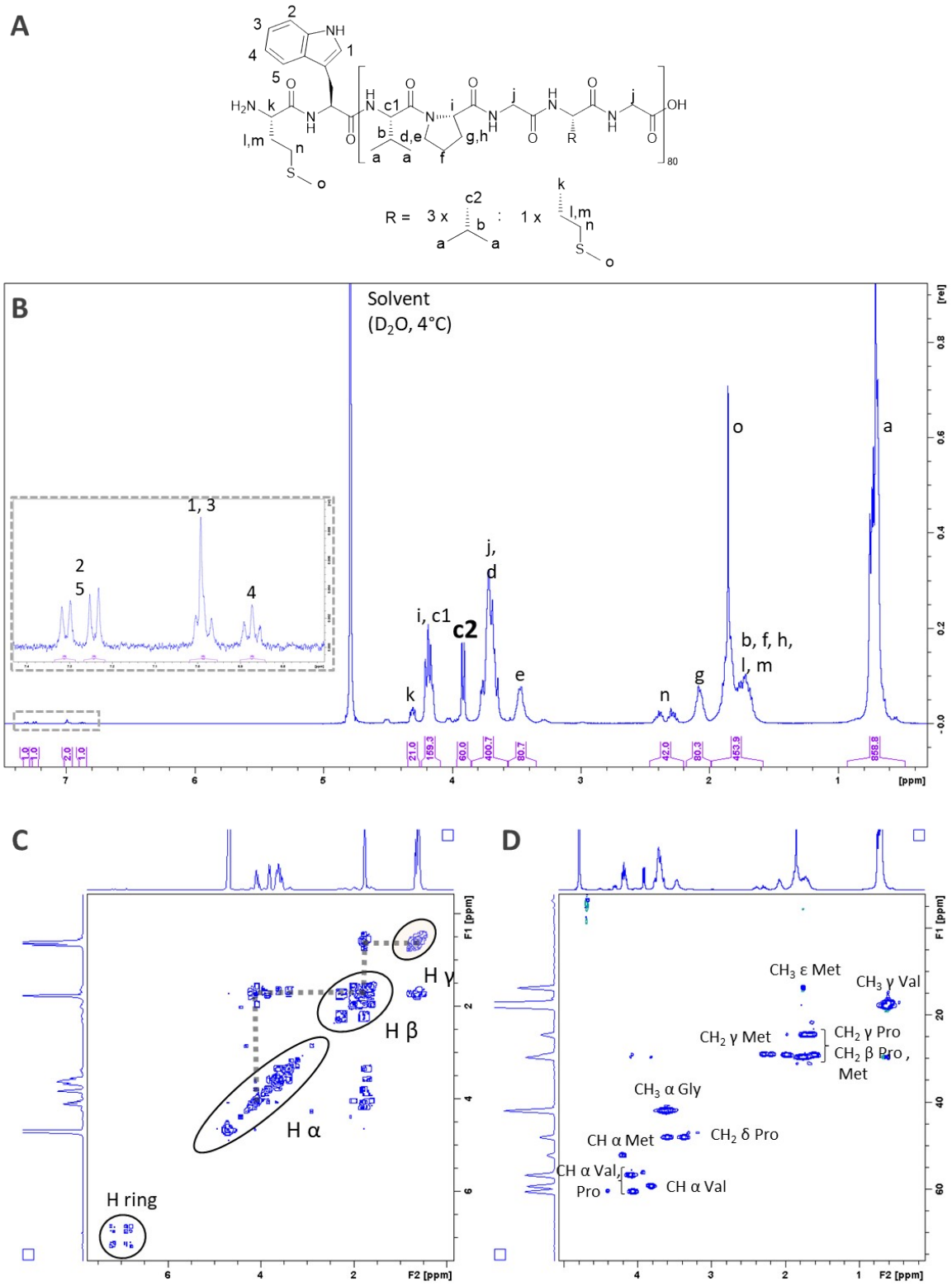

Figure S3: *NMR characterization of ELP[M_1V_3-80] (D_2O, 4°C, 400 MHz). (A) Chemical structure and proton notation. (B) ^{1}H NMR spectrum. Reference for integration: c2 (CH Valine VPGVG, 60 H). (C) COSY spectrum. Coupling between protons in α, β, and γ of the peptide chain and on the aromatic ring. (D) HSQC spectrum. Coupling between adjacent protons and carbons. Similar analyses were performed on ELPn60 and ELPn100. (Data not shown)*

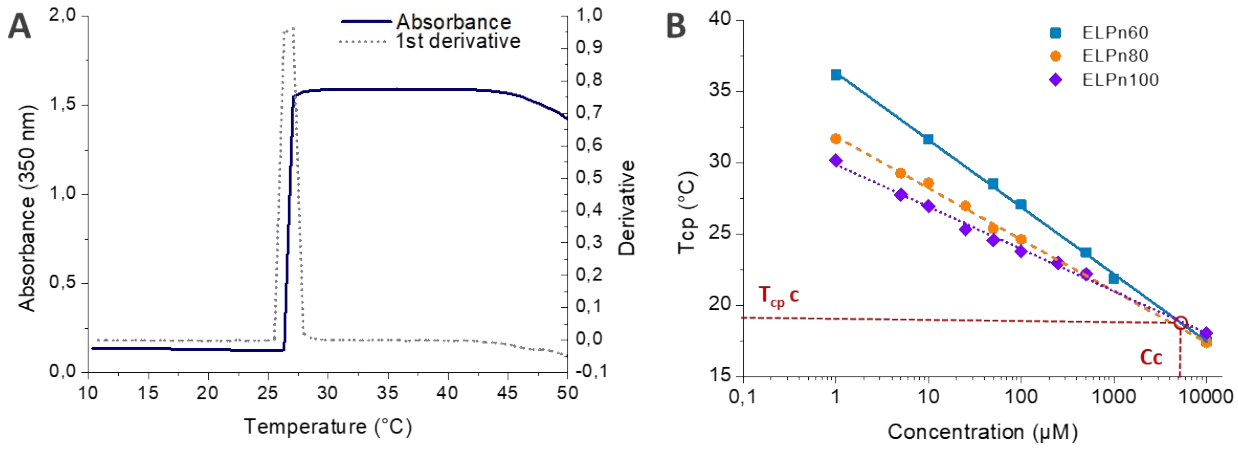

Figure S4: *Characterization of the thermo-responsive properties. (A) Turbidimetry measurement for a solution of ELPn60 at 100 µM in saline TRIS HCl buffer. The T_{cp} is defined as the temperature at the maximum of the 1st derivative. (B) Evolution of T_{cp} with molar concentration for ELPn60, ELPn80, and ELPn100. Fitting with a model "$T_{cp} = a - b * \ln(C)$"[2]. The intersection point between the 3 curves provides the coordinates (C_c and $T_{cp,c}$) of the LCST of the ELP[M_1V_3-n] family.*

ELP[M_1V_3-n]	M_w th (g/mol)	Recombinant production		Molar Mass			Thermoresponsiveness		
		Mean Yield (mg/L)	Number of productions	M_n SEC (g/mol)	M_w SEC (g/mol)	M_w MALDI (g/mol)	T_{cp} at 100 µM (°C)	T_{cp} at 1 µM (°C)	slope b
n = 60	**25,385**	110 (± 25)	10	27,670	28,760	25,417	27	36	2.05
n = 80	**33,700**	117 (± 4)	4	35,830	37,570	33,788	25	32	1.57
n = 100	**42,000**	73 (± 44)	8	44,550	46,130	42,122	24	30	1.29

Figure S5: *Table summarizing the molar mass and the thermo-responsive properties of ELP[M_1V_3-n] (n = 60, 80, 100). Mw th: theoretical molar mass. Slope b: value of the slope in the fitting model "$T_{cp} = a - b * \ln(C)$". b = k/n, with k a positive constant and n the number of repetitions of the pentapeptide.*

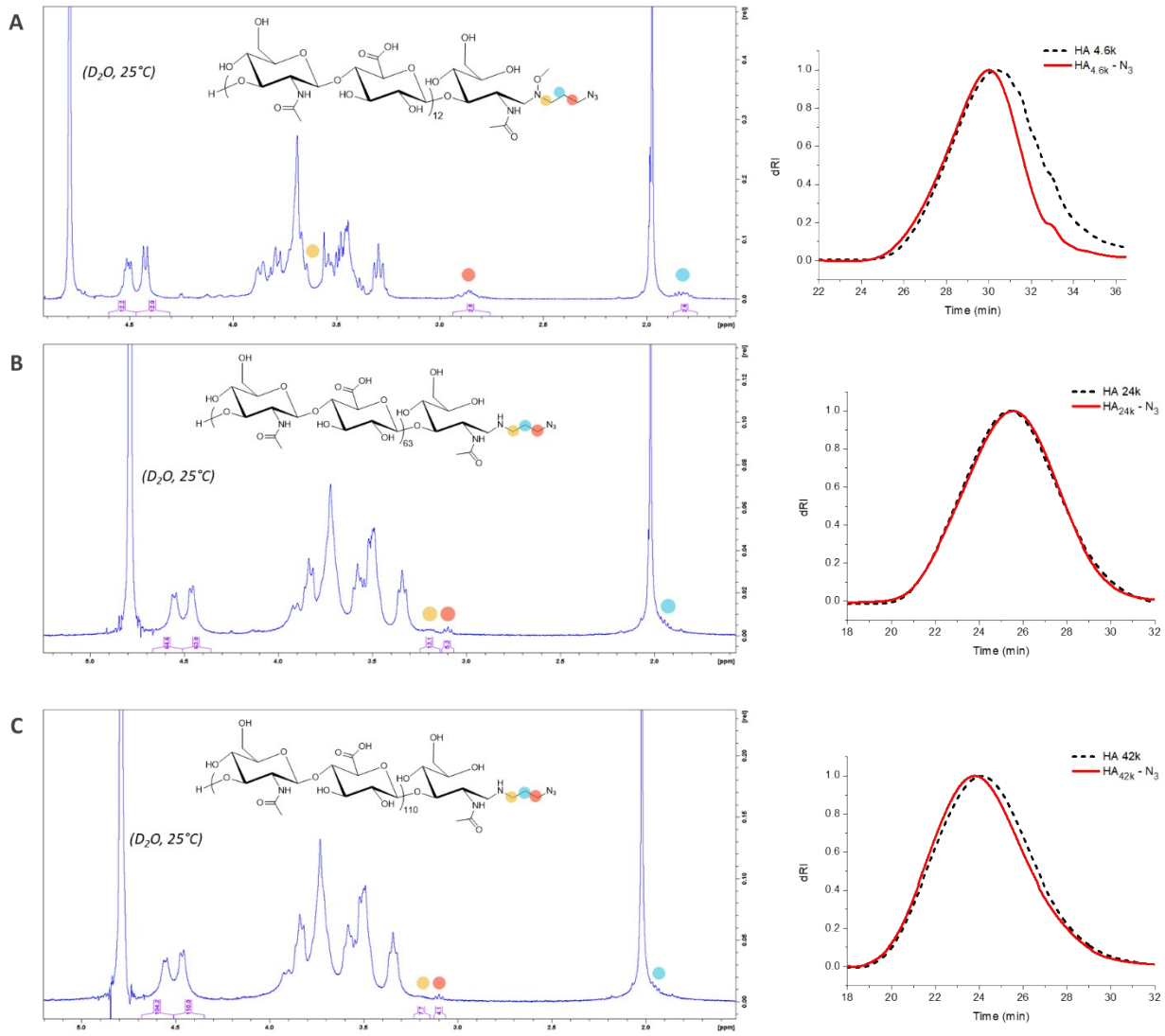

Figure S6: *Characterization of $HA_{4.6k}$-N_3 (A), HA_{24k}-N_3 (B) and HA_{42k}-N_3 (C). Left: ^1H NMR spectra (D_2O, 25°C, 400 MHz). Right: SEC chromatograms (phosphate buffer) of HA (black) and HA-N_3 (red).*

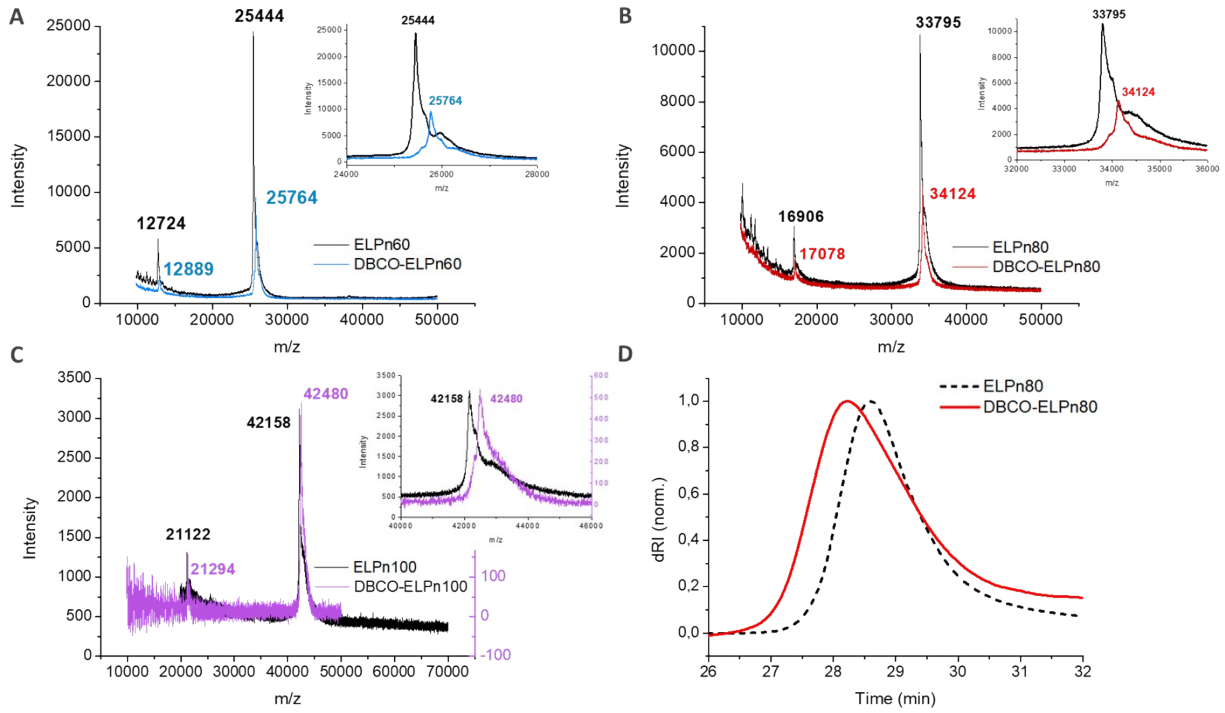

Figure S7: *Characterization of ELP before (black) and after (colored) functionalization with DBCO. A, B, C: MALDI spectrograms for (DBCO-)ELPn, with n = 60, 80,100 respectively. Theoretical m/z shift = +315 g/mol. D: SEC chromatograms of ELPn80 (black) and DBCO-ELPn80 (orange) in phosphate buffer.*

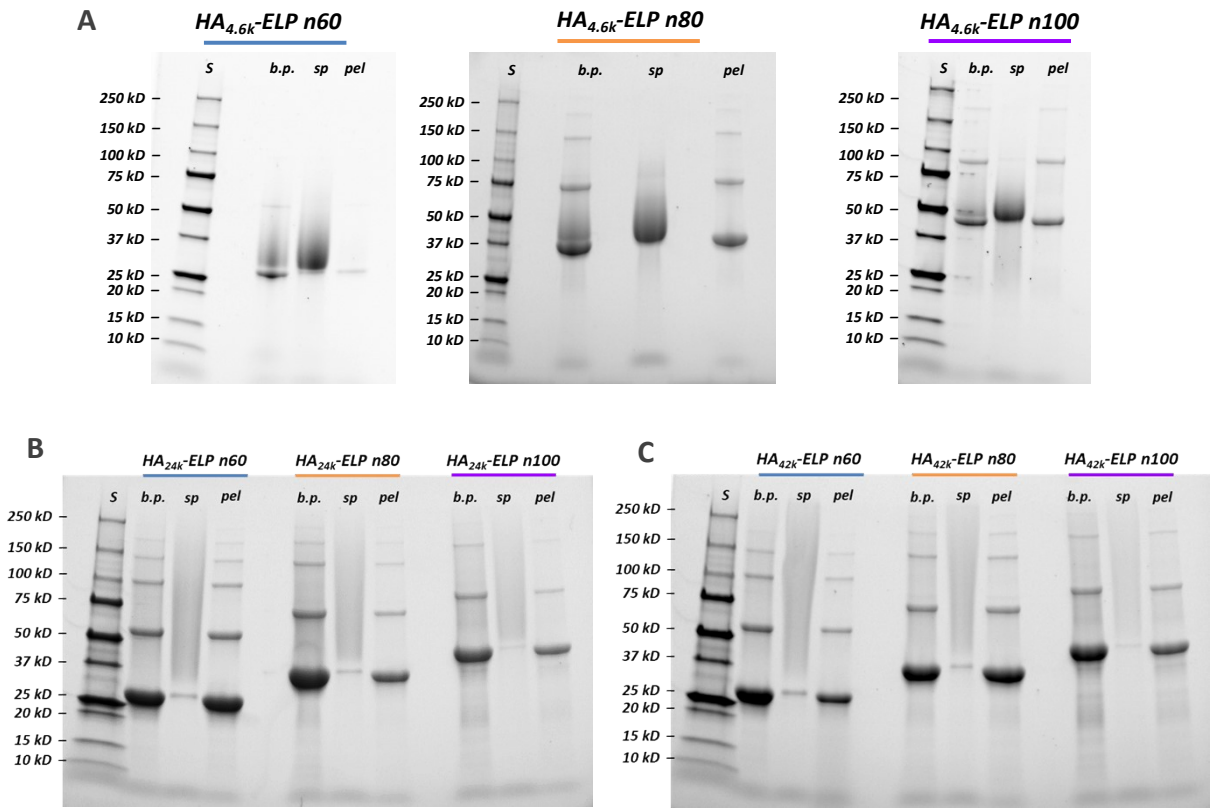

Figure S8: *Characterization of the entire library of bioconjugates by SDS-PAGE. A: $HA_{4.6k}$-ELPn (n=60,80,100); B: HA_{24k}-ELPn (n=60,80,100); C: HA_{42k}-ELPn (n=60,80,100). S: protein size markers; b.p.: reaction medium before purification; sp: supernatant; pel: pellet. B: samples in phosphate buffer. To note, the figure for $HA_{4.6k}$-ELPn80 (panel A, figure in the middle), provided in the main manuscript (Figure 2A), is here shown again for comparison to the other bioconjugates.*

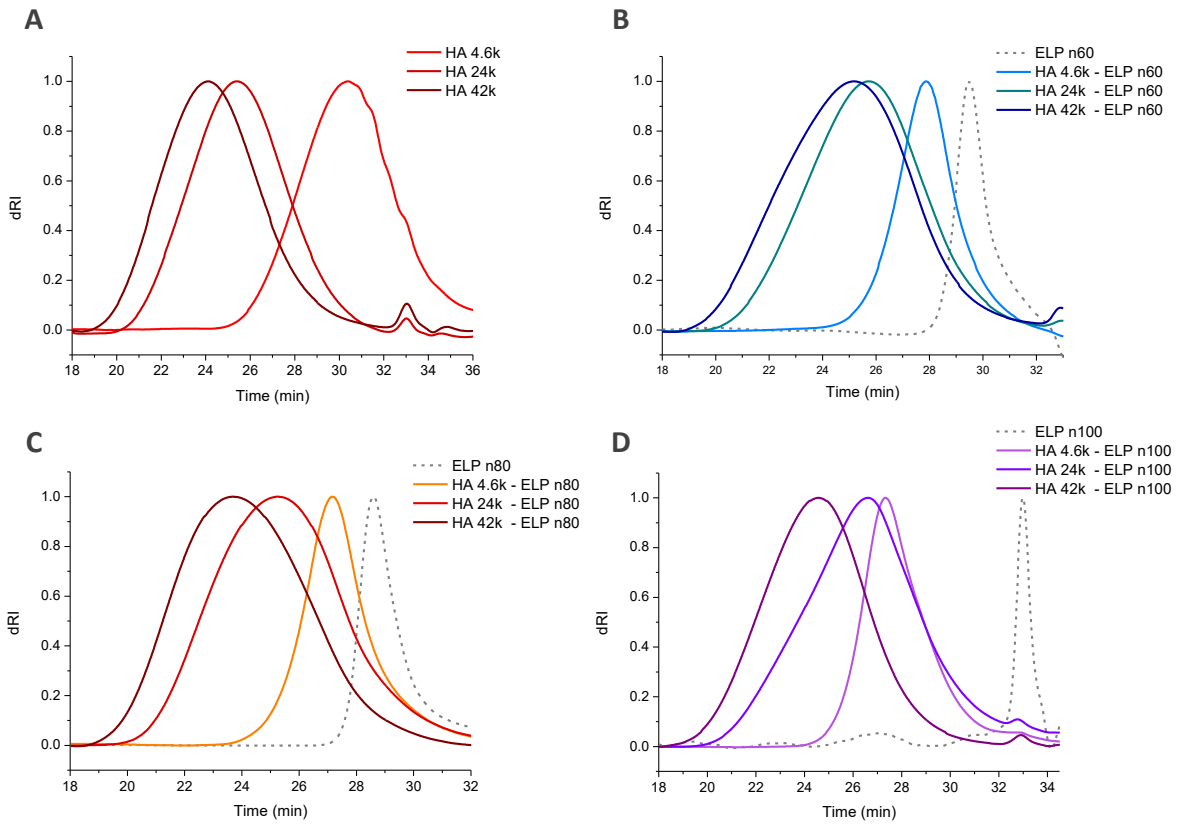

Figure S9: *Characterization of the entire library of bioconjugates by SEC. Normalized SEC chromatograms of A: commercial $HA_{4.6k}$, HA_{24k}, and HA_{42k}; B: HA_{xk}-ELP60; B: HA_{xk}-ELP80; D: HA_{xk}-ELP100. In panels B, C, and D, the chromatogram of the starting ELPn (n=60,80,100) is also included (grey dash). Samples in phosphate buffer.*

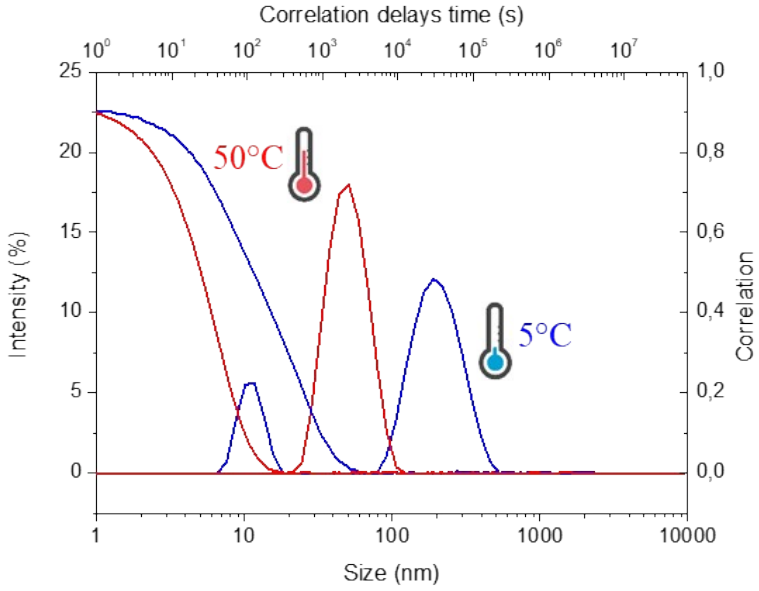

***Figure S10**: Characterization of the self-assembly by DLS for a solution of bioconjugates $HA_{4.6k}$-ELPn80 at 78 µM in PBS. Size distributions and associated correlograms at T<CMT (5°C) and T>CMT (50°C).*

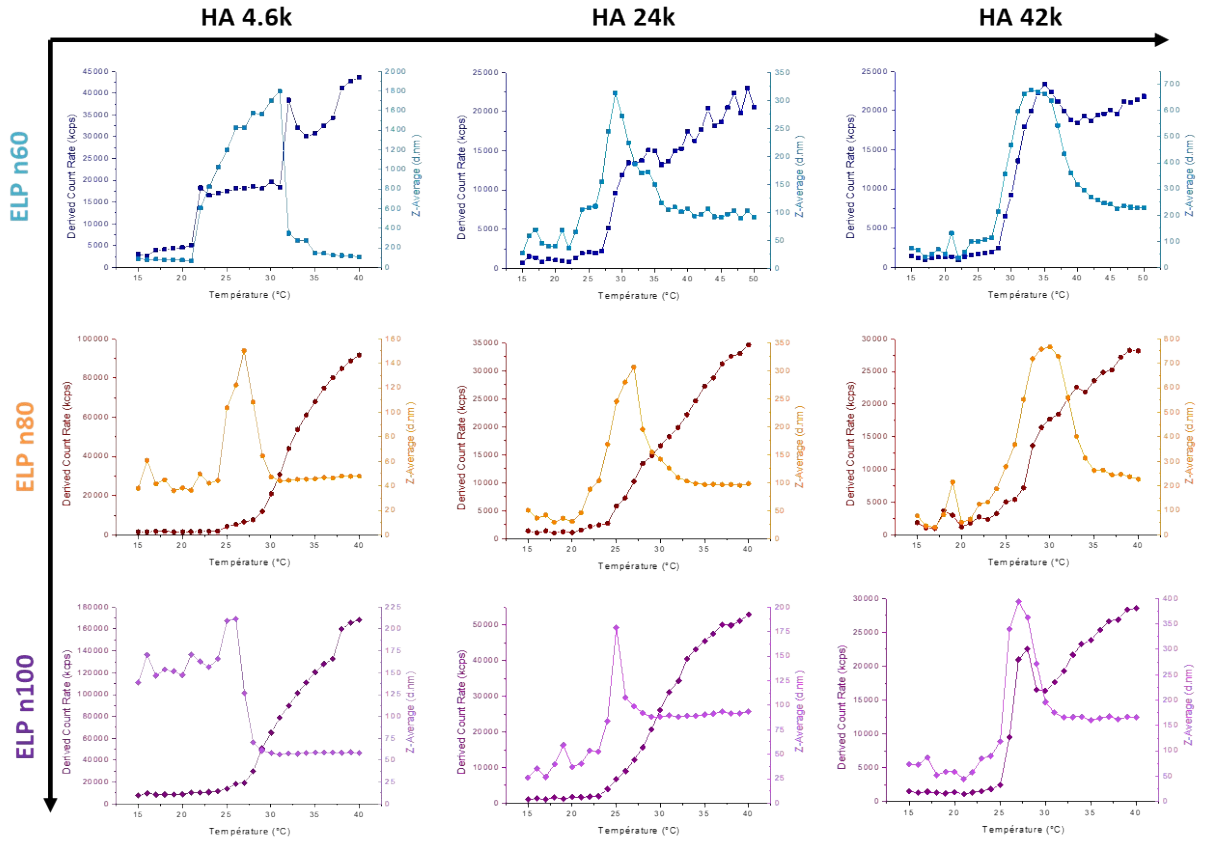

***Figure S11:** Critical Micellar Temperature (CMT) determination by DLS analysis for the whole library of HA-ELP bioconjugates (78 µM in PBS). Evolution of the derived count rate (dark, left axis) and of the z-average (light, right axis) with temperature (heating ramp: 15°C-40°C).*

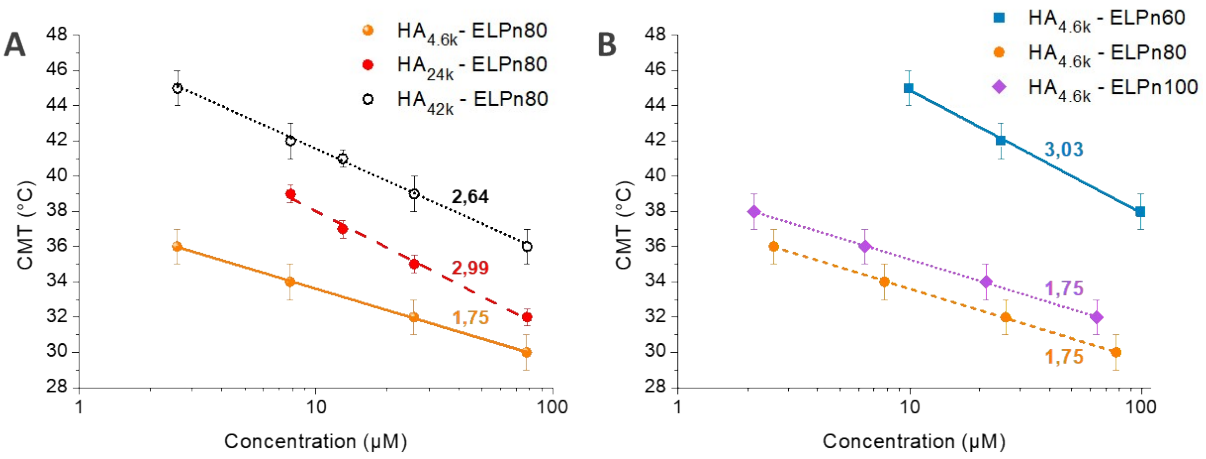

Figure S12: *Evolution of the CMT measured by DLS with the concentration in function of the structure of the bioconjugates. A: varying HA block size. B: varying ELP block size. Fit: CMT = a – b * Ln (C). Values of the slope b displayed on the graphs.*

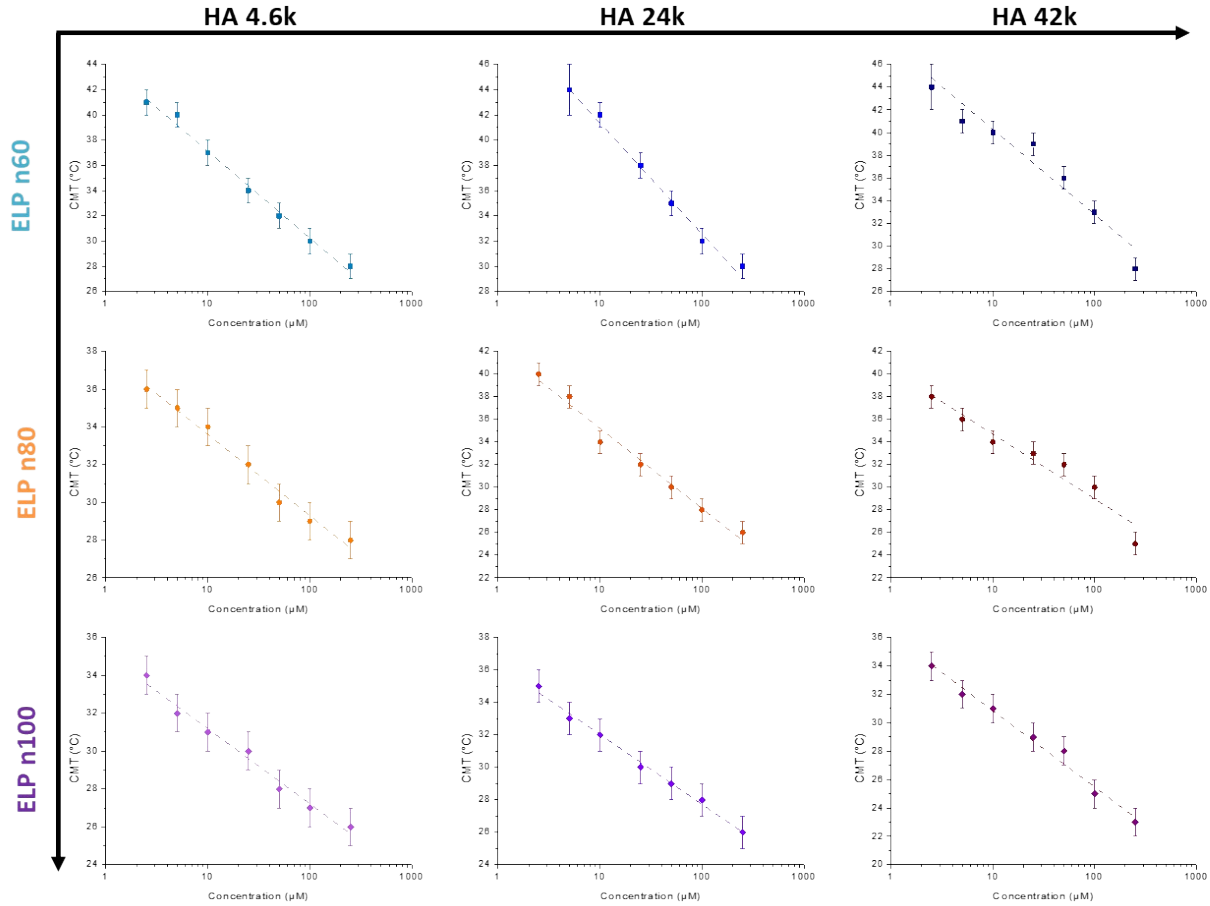

Figure S13: *Evolution of the CMT determined by fluorescence spectroscopy with concentration for the whole library of HA-ELP bioconjugates.*

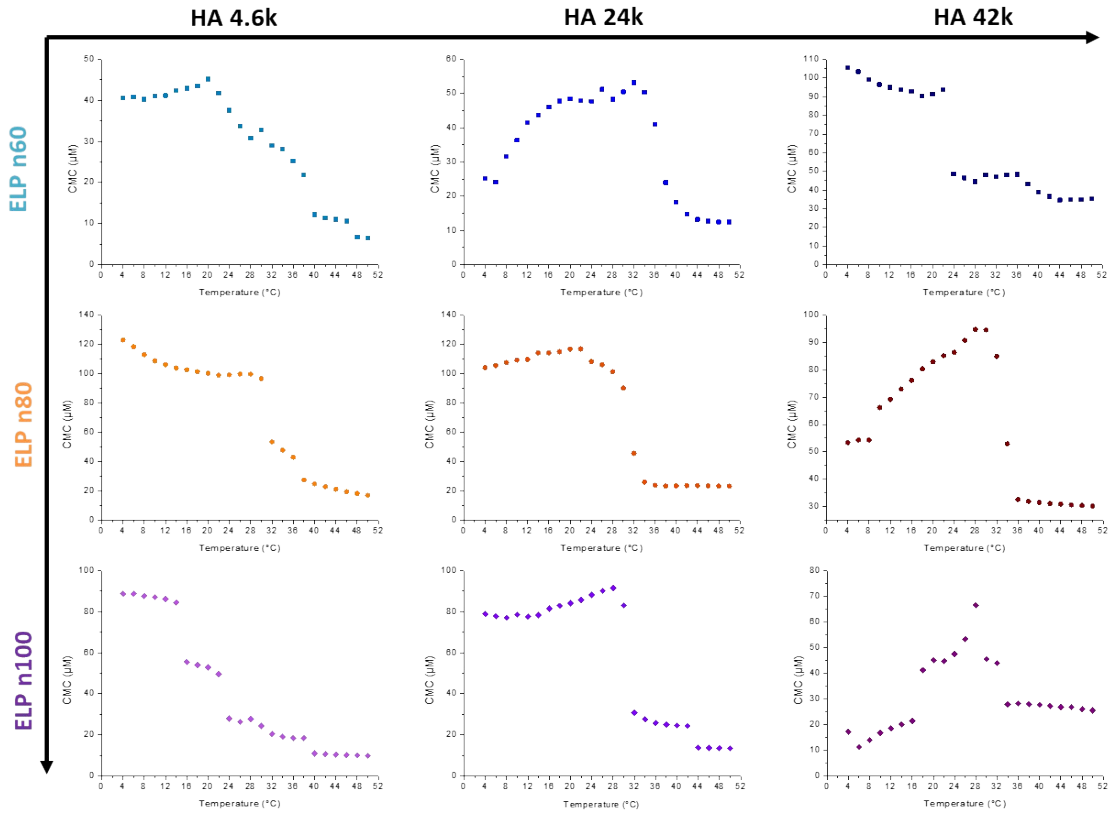

Figure S14: *Evolution of the CMC determined by fluorescence spectroscopy with temperature for the whole library of HA-ELP bioconjugates.*

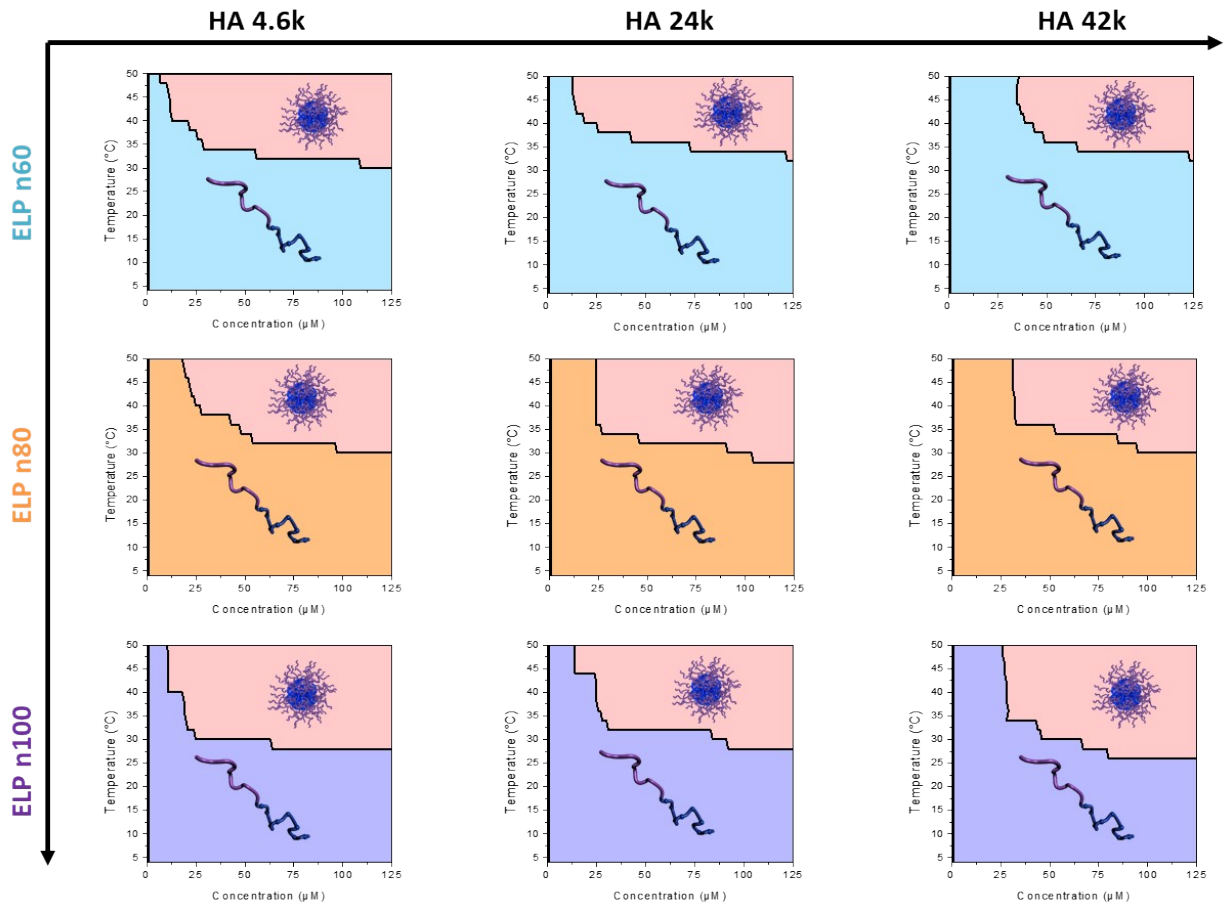

Figure S15: *Phase diagrams of the whole library of HA-ELP bioconjugates. Bottom zone: free chains. Red zone: self-assembled nanoparticles. Coacervate phase not shown.*