Noncovalent modulation of the inverse temperature transition and self-assembly of elastin-*b*-collagen-like peptide bioconjugates

Supporting Information

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Experimental Section

Materials

Fmoc-protected amino acids including Fmoc-propargyl glycine, Rink amide MBHA resin, N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), and piperidine for solid-phase peptide synthesis were purchased from AAPPTEC Inc. (Louisville, KY). HPLC-grade acetonitrile, dimethylformamide (DMF) and copper wire were purchased from Fisher Scientific (Fairlawn, NJ). Copper(I) Acetate, 4-azidobutanoic acid, N-methyl-2-pyrrolidone (NMP), trifluoroacetic acid (TFA), triisopropylsilane (TIS), triethylamine (TEA), diisopropylethylamine (DIEA) and deuterated DMSO were purchased from Sigma-Aldrich (St. Louis, MO).

Peptide synthesis

A collagen-like peptide with the sequence $(GPO)_4GFOGER(GPO)_4GG$ and an elastin-like peptide with sequence $(VPGFG)_6G'$ (G': propargyl glycine) were synthesized via traditional solid-phase peptide synthesis methods (SPPS) using a Focus XC automatic peptide synthesizer (AAPPTec Inc., Louisville, KY). Rink amide MBHA resin with a loading capacity of 0.52 mmol/g was used for the synthesis. The amino acids were activated for coupling with HBTU in the presence of 2 M diisopropylethylamine (DIEA) in NMP. Deprotection of the Fmoc group was conducted using 20% piperidine in DMF. One-hour coupling cycles were used for all the residues. 4-azidobutanoic acid was manually attached to the N-terminus of the CLP on resin. Double coupling with 4:1 amino acid/resin ratio was used for the conjugation. Cleavage of the peptides from the resin was conducted in 95:2.5:2.5 (v:v:v) trifluoroacetic acid (TFA) /triisopropylsilane (TIS) /water for 3 hours. The TFA was mostly evaporated and the cleaved peptide was precipitated in cold ether. The peptide was then redissolved in water and lyophilized.

Crude peptides were purified via reverse-phase HPLC (Waters Inc., Milford, MA) on a Waters Xbridge BEH130 Prep C-18 column. The mobile-phase comprised gradients of degassed deionized water with 0.1% TFA and acetonitrile with 0.1% TFA, at a flow rate of 21 ml/min. Peptide was detected by a UV detector at 214 nm; fractions with product were collected and lyophilized. The molecular weight of the peptides was confirmed via electrospray ionization mass spectrometry (ESI-MS, AutospecQ, VG Analytical, Manchester, UK) and the purity of the peptide was confirmed via analytical scale, reverse-phase HPLC (Waters 2996; Symmetry C18, 3.5 µm, 4.6 x 75 mm).

ELP-CLP conjugate synthesis

The synthesis of the conjugate was performed via copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) "click" reaction.[1] Solutions of CLP (8.57 mg, 3 µmol) in 0.5 mL anhydrous DMF, ELP (9.12 mg, 3 µmol) in 0.5 mL anhydrous DMF, and Cu(I) acetate (0.25 equiv. to alkyne) in 0.5 mL anhydrous DMF were added to a nitrogen-purged vial. DMF was employed for the reaction because in polar aprotic solvents, the CLP and CLP-ELP conjugate are not able to form stable triple helical conformations, which we expected would improve the efficiency of the reaction. In addition, DMF is also a good solvent for the ELP as well, so none of the reactants or products precipitate during the reaction. The mixture was stirred at 80 °C under nitrogen for 24 h. After reaction, the resulting hybrid copolymer was isolated into a 5-fold volume of cold diethyl ether and redissolved in water to remove the catalysts. The diblock solution was then dialyzed against water for 7 days to remove the residual catalyst. A dialysis membrane with MWCO of 1000 Da

was used for the process. The product was then collected and lyophilized at nearly 100% yield, indicating that the reaction conditions do not degrade the peptides. Characterization of the products (below) also indicated the stability of the peptides under the coupling reaction conditions. The ELP-CLP diblock showed self-assembly and formed aggregates in aqueous solutions, and similar aggregation was also observed in acetonitrile-based solvents. HPLC therefore could not be performed for the conjugate, and GPC (in organic solvents)/NMR/FTIR were used to verify the identity and purity of the diblock products.

Gel Permeation Chromatography (GPC)

GPC was performed in trifluoroethanol (TFE) with 0.02 M sodium trifluoroacetate at 40 °C using an Agilent 1200 system equipped with an isocratic pump operated at 1 mL/min, one 50 mm x 8 mm PSS PFG guard column (Polymer Standards Service), three 300 mm x 7.5 mm PSS PFG analytical linear M columns with 7 µm particle size (Polymer Standards Service), and a refractive index detector. The system was calibrated with PMMA standards. 100 µL sample was used for each injection.

Nuclear Magnetic Resonance Spectrometry (NMR)

^{1}H NMR spectra were recorded under standard quantitative conditions on a Bruker AVIII spectrometer operating at 600 MHz, using at least 64 scans. All samples were dissolved in deuterated dimethyl sulfoxide (δ (d6-DMSO) = 2.50 ppm) at a concentration of 2 mg/mL. The resulting spectra were analyzed using Mnova software (Mestrelab Research, Santiago de Compostela, Spain).

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were collected using a Thermo Nicolet Nexus 670 (Thermo Scientific, Waltham, MA, USA) spectrometer with a DuraSamplIR II ATR accessory (Smiths Detection, Danbury, CT, USA). The peptide samples were added as solids onto the silicon ATR crystal and gently pressed down during data acquisition (128 scans at 4 cm^{-1} resolution from 1000 - 4000 cm^{-1}). A background of the clean silicon crystal in air was subtracted from all sample spectra.

Circular Dichroic Spectroscopy (CD)

Characterization of the secondary structure of the CLP domain was conducted via circular dichroic spectroscopy (Jasco 810 circular dichroism spectropolarimeter, Jasco Inc., Easton, MD, USA). Either CLP, ELP or ELP-CLP conjugate was dissolved at a concentration of 100 µM in PBS (10 mM, pH 7.4, 137 mM NaCl and 2.7 mM KCl) and incubated overnight before measurement. The CD spectra were recorded using quartz cells with a 0.2 cm optical path length.

Full wavelength scans were collected to study the conformation of the peptide domain at selected temperatures. The sample was incubated at each temperature for 10 min before measurement. The scanning rate was 50 nm/min, with a response time of 4 s. The wavelength scans were obtained from 200 nm to 250 nm and were recorded every 1 nm. In order to precisely measure the melting temperature of the CLP domain, variable temperature experiments were conducted at a constant wavelength of 225 nm with a 0.25 °C/min heating rate. Refolding kinetics were studied via temperature-jump experiments. The sample solution was incubated at 80 °C for 30

min followed by quenching to 5 °C in less than 2 minutes. The ellipticity at 225 nm as a function of time was monitored at 5 °C beginning right after the temperature jump. Although in the refolding experiment, the CLP triple helix did not reach a 100% folded state, a 4 hour refolding time was sufficient to allow accurate calculation of the refolding rate constants, as well as to provide a good comparison between the refolding behavior of the CLP and ELP-CLP diblock conjugate.

Dynamic Light Scattering (DLS)

Analysis of particle sizes in solution was conducted via dynamic light scattering (DLS) on a ZetaSizer Nano Series (Nano ZS, Malvern Instruments, U.K.) at a scattering angle of 173°, and data fitting using the cumulant method. CLP, ELP and ELP-CLP conjugate samples were all prepared at 1 mg/mL in deionized water. A CLP (0.5 mg/mL) and ELP (0.5 mg/mL) physical mixture dissolved in water, as well as ELP samples (1 mg/mL) in 0.1 M NaCl and 0.3 NaCl solution were also prepared as controls. All samples were incubated at 4 °C overnight before measurement. The lower critical solution temperature (LCST) of the ELP and ELP-CLP conjugate was assessed by measurement of the average size of particles at temperatures from 5 °C to 80 °C, at an interval of 2 °C. Samples were incubated at each temperature for 2 minutes before measurements. The LCST was assigned as the temperature at which the intensity of scattered light began to increase. For particle size distribution studies at 25 °C, 37 °C, 50 °C, 65 °C and 80 °C, each sample was incubated for 10 min at the desired temperature before measurements. The reported data represent an average of at least three measurements.

Transmission Electron Microscopy (TEM)

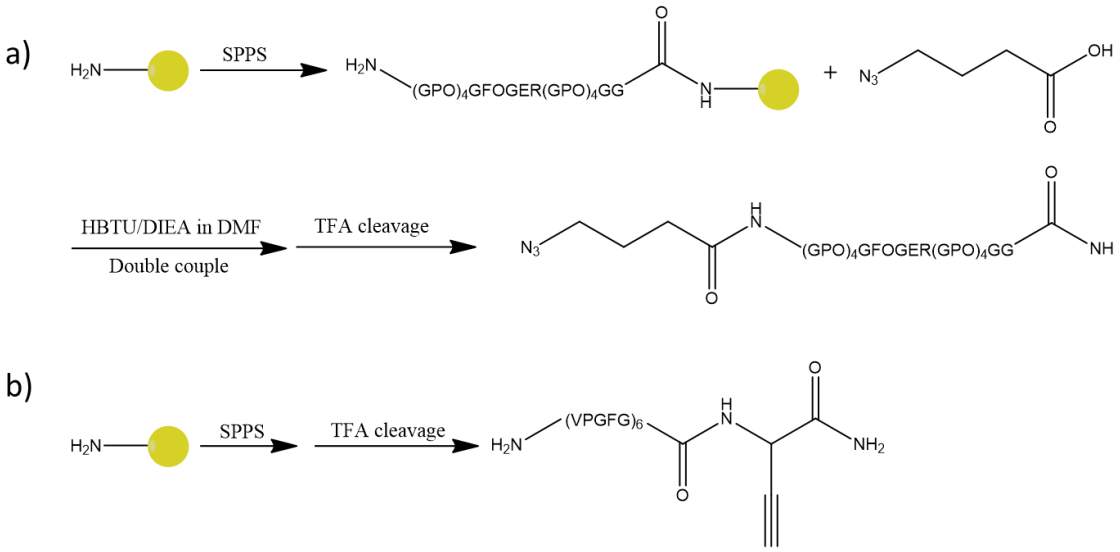
Samples for TEM were prepared on carbon-coated copper grids (CF300-Cu, Electron Microscopy Sciences Inc.). The grids, pipette tips, and samples were incubated in an isothermal oven (VWR Signature™ Forced Air Safety Ovens, VWR Inc.) at desired temperature (25 °C, 37 °C, 50 °C, 65 °C and 80 °C) for at least 30 min before sample preparation, which was also conducted in the oven. ELP-CLP diblock sample was dissolved in water at concentration of 1 mg/mL (consistent with DLS exeperiments and to prevent precipitation of the PTA stain). 5 µL of the sample solution was drop cast on the grid and blotted after 60 seconds. For staining, 1% phosphotungstic acid (PTA) (pH adjusted to 7.0 using 1 M NaOH) as a negative stain was used. 3 µL of the PTA solution was drop cast on the grid and blotted after 10 seconds. The sample was allowed to dry in the oven at the desired temperature for 30 minutes and then was air-dried for 2 hours. TEM images were taken on a JEM-3010 TEM (JEOL USA Inc., Peabody, MA) at an acceleration voltage of 200 keV.

Cryo Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM was performed in a Tecnai 12 microscope operated at 120 kV. 3 µL of the diblock sample solution at 1 mg/mL dissolved in water was placed on a 300 mesh quantifoil R1.2/1.3 film supported on a copper holey carbon grid (SPI supplies inc., West Chester, PA) within a Vitrobot vitrication system (FEI Inc. Hillsboro, OR). The sample was blotted and quickly plunged into a liquid ethane reservoir cooled by liquid nitrogen. The vitrified samples were transferred to a Gatan 626 cryo-holder and cryo-transfer stage cooled by liquid nitrogen. During observation of the vitrified samples, the cryo-holder temperature was maintained below -176 °C. The images were recorded digitally with a Gatan CCD camera.

Conjugate synthesis and characterization

Both peptides were synthesized via traditional Fmoc-based, solid-phase peptide synthesis methods (SPPS) (Scheme S1). An azide was introduced to the N terminal of the CLP and an alkyne group was introduced to the C terminal of the ELP to allow facile conjugation of the two peptides via CuAAC "click" reaction. After purification with reverse-phase HPLC, peptides with purity greater than 90% was obtained (Figure S1). The molecular weight of both peptides was verified via ESI-MS (Figure S2 and Figure S3).For CLP, Mw = 3039 Da, m/z = 1520.6 $[(M + 2H)^{2+}$, calcd 1520.5], m/z = 1014.3 $[(M + 3H)^{3+}$, calcd 1014.0]. For ELP, Mw = 2856 Da, m/z = 1429.3 $[(M + 2H)^{2+}$, calcd 1429.0], m/z= 960.7 $[(M + 2H+Na)^{3+}$, calcd 960.3].



Scheme S1. Solid phase peptide synthesis and molecular structure of: a) azide functionalized collagen-like peptide; b) alkyne functionalized elastin-like peptide

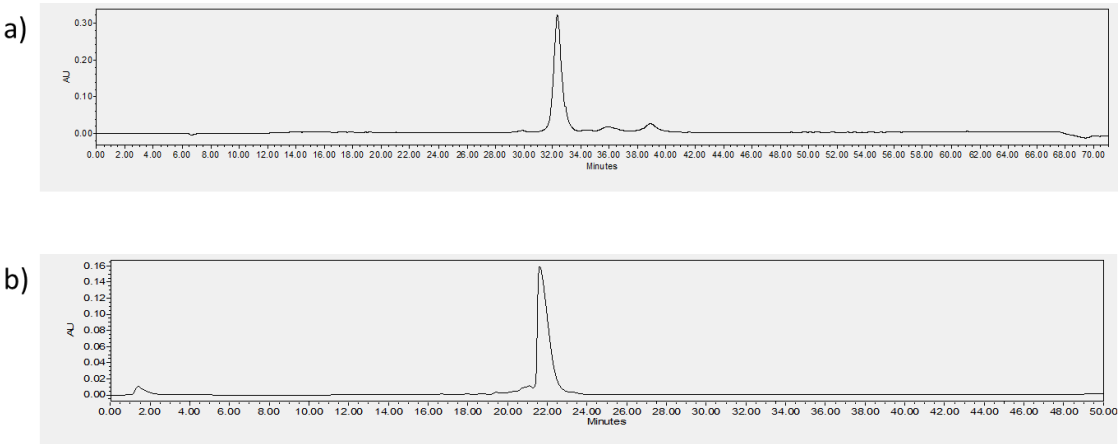


Figure S1. RP-HPLC trace of purified peptides: a) collagen-like peptide; b) elastin-like peptide

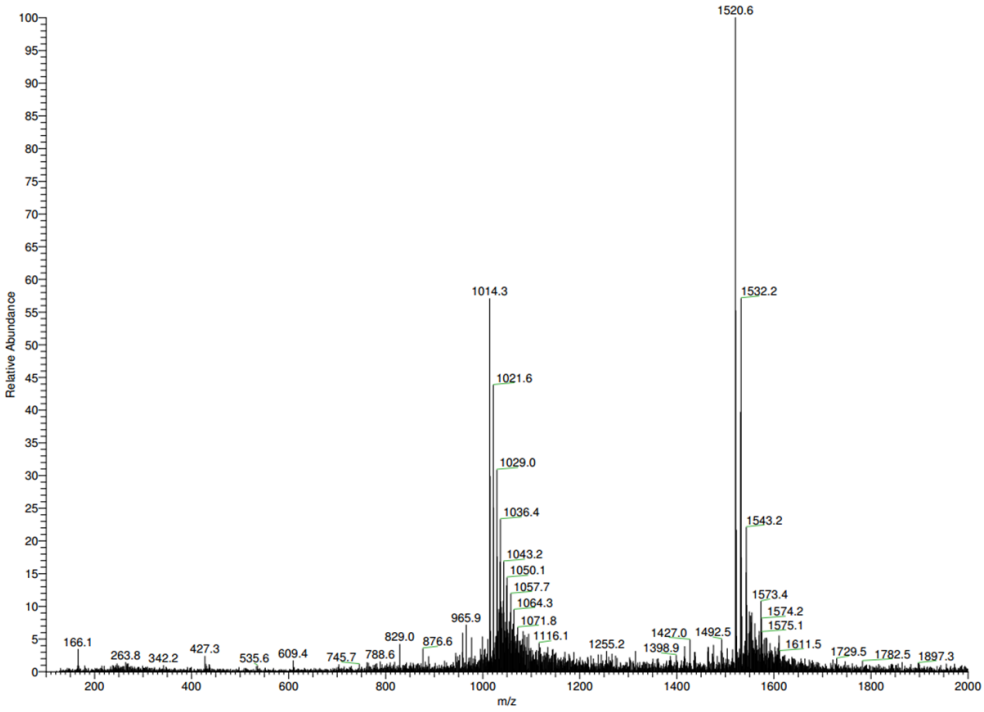


Figure S2. ESI-MS of purified CLP.

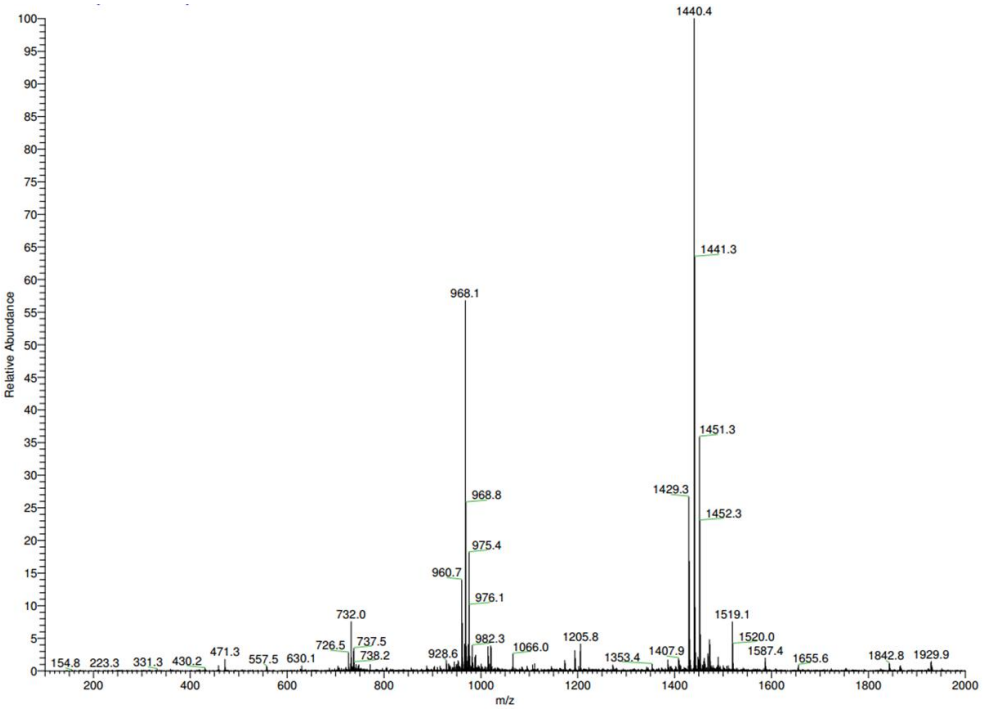


Figure S3. ESI-MS of purified ELP.

The alkyne functionalized ELP was conjugated to the azide functionalized CLP via CuAAC "click" reaction, employing DMF as a solvent in order to ensure the solubility of all reactants and the ELP-CLP product. The product was collected in nearly 100% yield, indicating the lack of peptide degradation under the reaction conditions. As discussed above, self-assembly and aggregation of the ELP-CLP conjugates in aqueous solutions and in acetonitrile-based solvents precluded characterization of the ELP-CLP conjugates via HPLC. Therefore, GPC (in organic solvents), ^{1}H NMR, and FTIR were used to verify the identity and purity of the diblock products. The molecular weight distribution of purified products was studied via gel permeation chromatography (GPC) conducted using trifluoroethanol (TFE) as the mobile phase (Figure S4). The traces shown were obtained using refractive index detection and were normalized to give a better comparison between the diblock and starting material. As shown in Figure S4, the elution time of the diblock was clearly shifted to the higher molecular weight region compared with the CLP or ELP starting material. The results suggest successful conjugation of the two building blocks and the product peak with a negligible shoulder in the lower molecular region indicates the click reaction was complete and successful removal of excess starting material. An estimation of molecular weight from a calibration employing linear poly (methyl methacrylate) (PMMA) standards yielded a Mw of 11.2 kDa of the ELP-CLP diblock, consistent with the addition of Mw of the ELP 4.8 kDa and the CLP 6.7 kDa. The molecular weights were larger than anticipated, likely due to the potential physicochemical differences between the peptides and PMMA standards, which would affect their mobility and/or hydrodynamic volume in the mobile phase. The smaller shoulder located at 27.5 min likely corresponds to the ELP-CLP trimer. Indeed, circular dichroism full wavelength scan experiments conducted on the diblock sample dissolved in TFE (Figure S5) suggested the diblock was partially folded in triple helical conformation under the same conditions as those under which the GPC was performed.

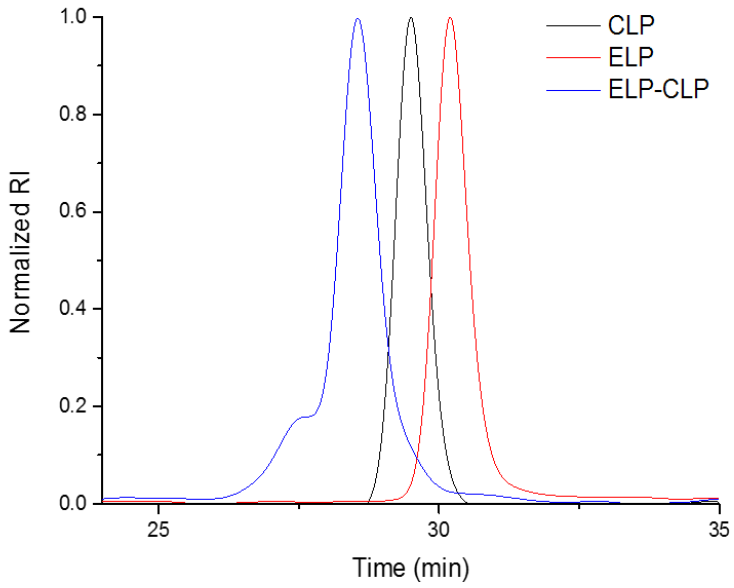


Figure S4. GPC trace of ELP-CLP diblock and peptide starting material.

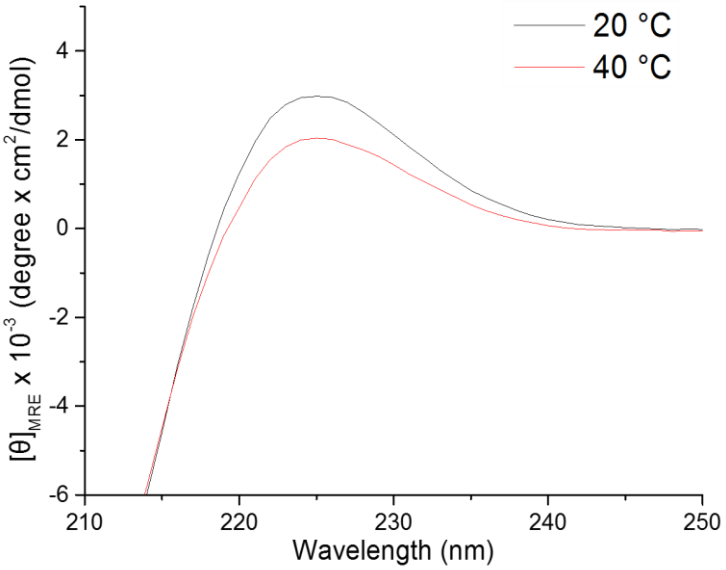


Figure S5. CD spectra showing the wavelength scans for the ELP-CLP diblock sample dissolved in TFE at 20 °C and 40 °C. Results suggested the diblock maintained a partially folded triple helical conformation when injected into the column for GPC experiments at 40 °C.

^{1}H NMR spectroscopy was utilized to confirm the presence of both the ELP and CLP domain in the diblock product (Figure S6). The spectrum of the ELP-CLP diblock, given in Figure S6c, showed all corresponding characteristic signals from the ELP as well as the CLP building block. A zoomed in spectrum of the diblock (Figure S7) suggested that the alkyne protons from ELP propargyl glycine vanished in the diblock sample, indicating complete consumption of the ELP starting material in the click reaction. FT-IR was used to track the azide group before and after the conjugation reaction. Spectra of the ELP and the azide functionalized CLP as well as the resulting ELP-CLP diblock are shown in Figure S8. The strong band at 2094 cm^{-1} is indicative of the azide end groups in the starting CLP. The band was not observed in the ELP-CLP diblock conjugate, indicating complete consumption of the CLP starting material in the click reaction. The NMR and FT-IR results, together with the higher MW suggested by GPC, suggest successful synthesis and purification of the diblock.

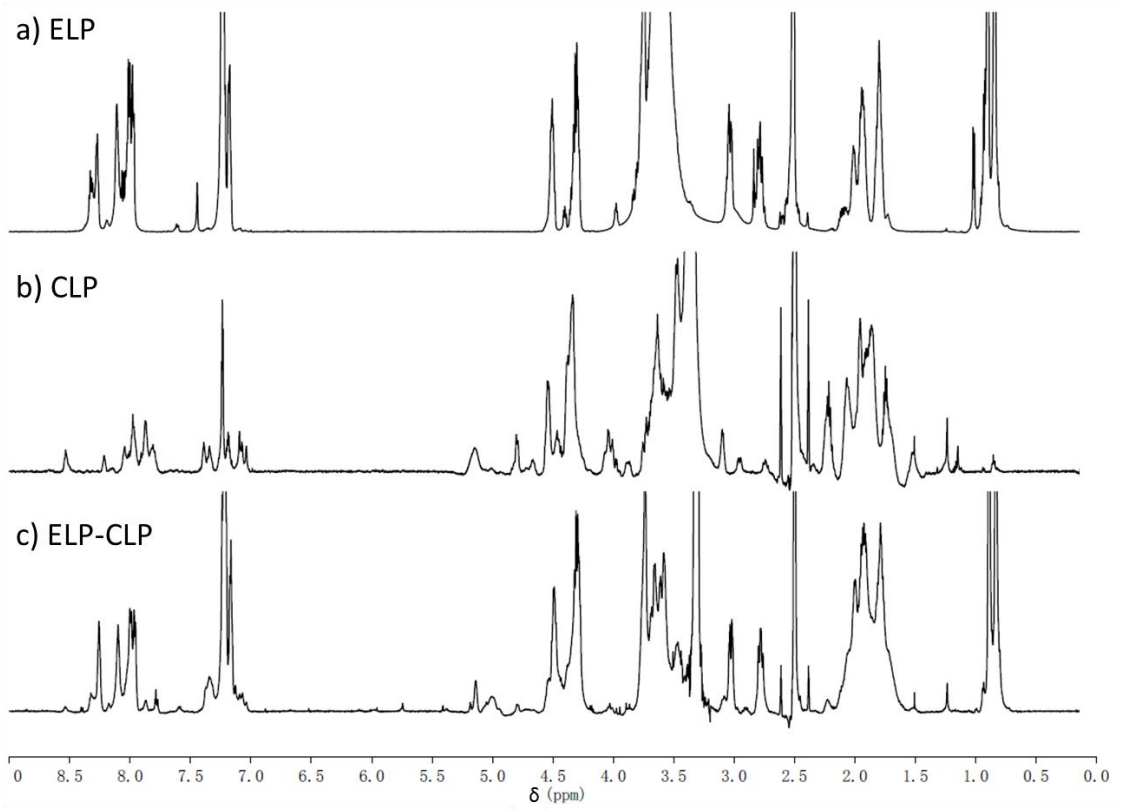


Figure S6. ^{1}H NMR spectra (600 MHz) of: a) elastin-like peptide; b) collagen-like peptide; c) ELP-CLP diblock in d_6-DMSO.

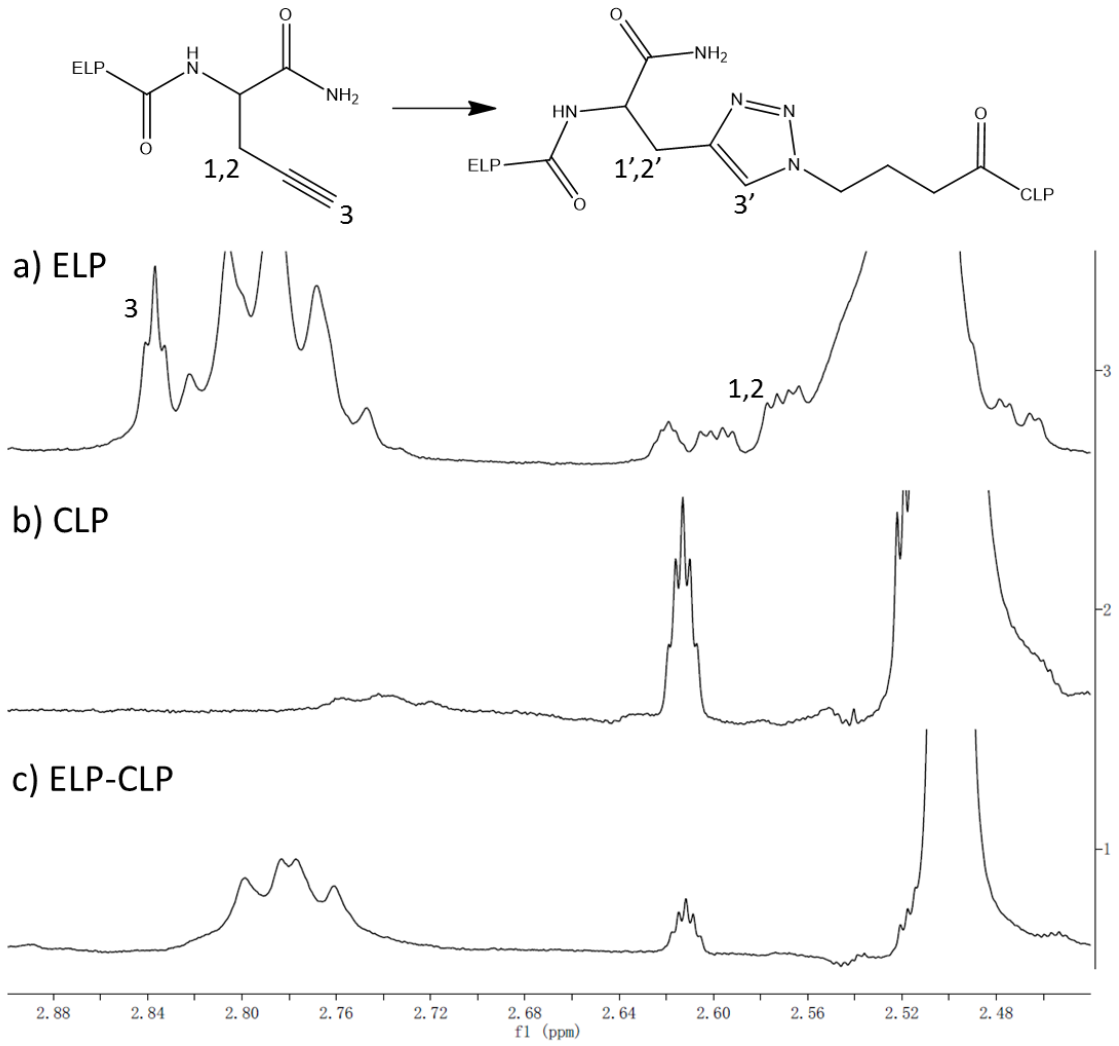


Figure S7. Expanded ^{1}H NMR spectra between 2.4 ppm and 2.9 ppm of: a) elastin-like peptide; b) collagen-like peptide; c) ELP-CLP diblock.

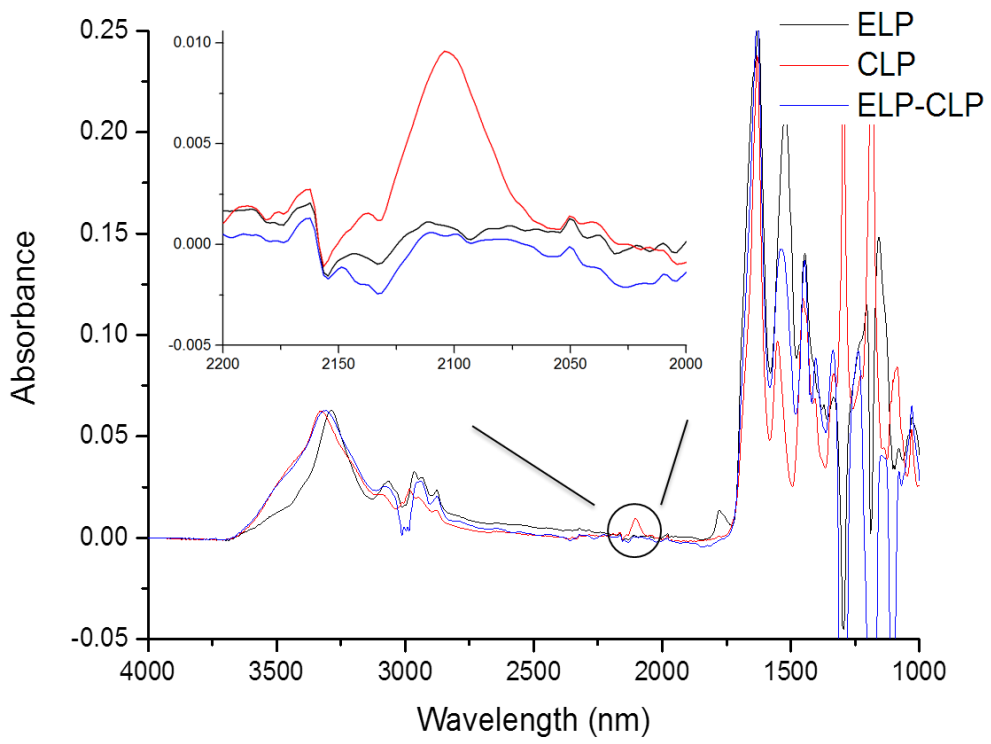


Figure S8. ATR-FTIR spectra of the ELP-CLP diblock and peptide starting materials.

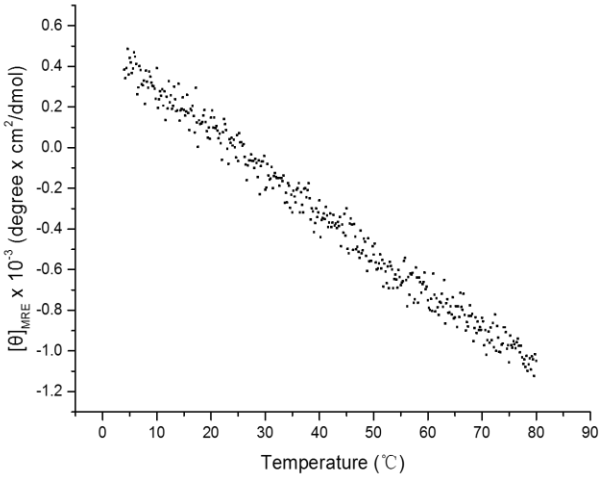


Figure S9. Thermal unfolding profile for ELP plotted as $[\theta]_{MRE225nm}$ versus temperature.It was subtracted from the diblock unfolding profile to remove the contribution from ELP domain.

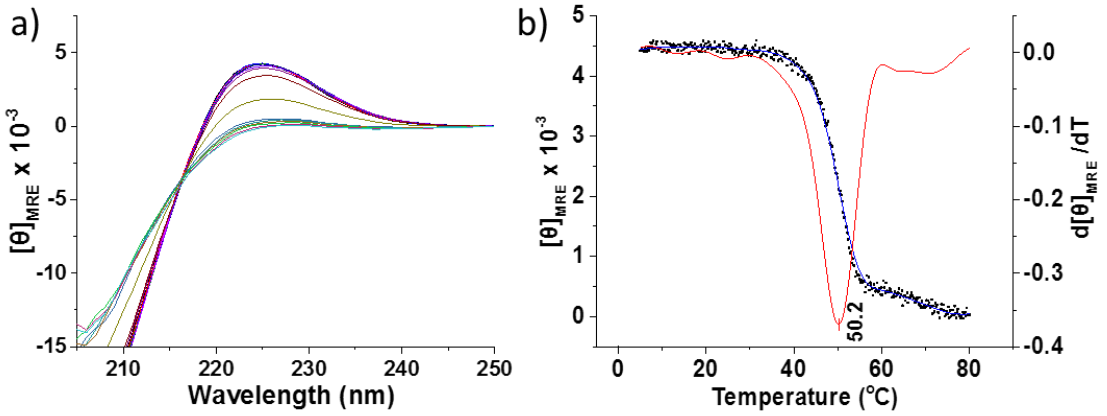


Figure S10. a) CD spectra showing the wavelength scans for the collagen like peptide; b) Blue curve with dots (Y axis on the left side): thermal unfolding profile for CLP; red curve (Y axis on the right side): first derivative of the unfolding curve with respect to temperature;

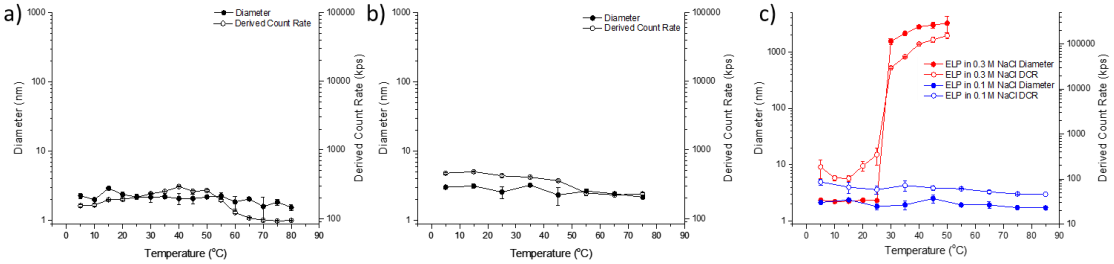


Figure S11. Hydrodynamic diameter of assemblies as a function of temperature upon heating. a) ELP at 1 mg/mL in water; b) ELP and CLP physical mixture at 0.5 mg/mL each in water; c) ELP at 1 mg/mL in NaCl aqueous solution

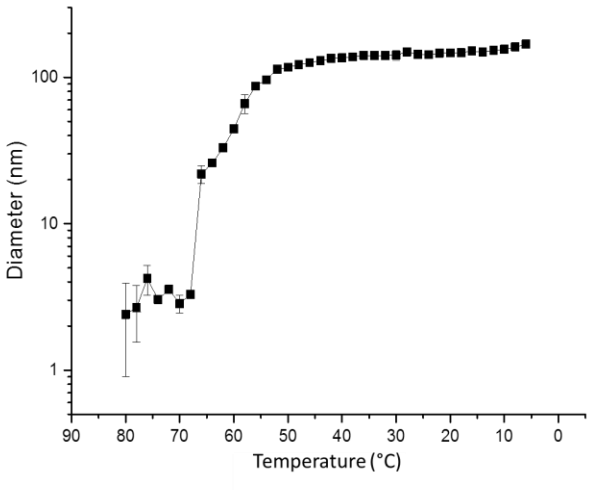


Figure S12. Hydrodynamic diameter of ELP-CLP diblock assemblies as a function of temperature upon cooling

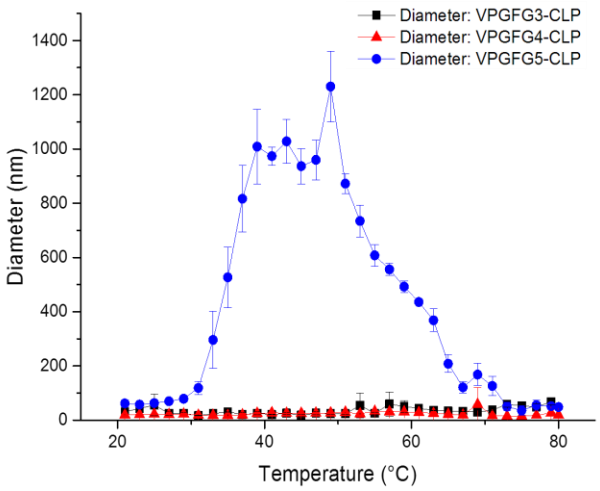


Figure S13. Hydrodynamic diameter of $(VPGFG)_{3\text{-}5}$-CLP diblock assemblies as a function of temperature upon heating

(1) Grieshaber, S. E.; Paik, B. A.; Bai, S.; Kiick, K. L.; Jia, X. Q. *Soft Matter* **2013**, *9*, 1589.