**Electronic Supplementary Information (ESI)**

Single-molecule mechanical unfolding experiments reveal a critical length for the formation of α-helices in peptides

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1. **PEG-b-PBLG synthesis**

PEG\textsubscript{112}-b-PBLG\textsubscript{90} was synthesized according to a protocol described elsewhere.\textsuperscript{S1,S2} Briefly, α-methoxy-ω-amino poly(ethylene glycol) (CH\textsubscript{2}O-PEG-NH\textsubscript{2}, 5000 g/mol, RAPP Polymere, Germany; 0.5 g, 0.1 mM) was dissolved in 2 mL dioxane, freeze-dried and dissolved in dry DMF (0.1 g/mL). In a glovebox, γ-benzyl-L-glutamate N-carboxyanhydride (Isochem, France; 2.6 g, 10 mM) was introduced into a flame-dried Schlenk flask and dissolved in anhydrous DMF (0.1 g/mL). This solution was added to the PEG solution under vacuum and the mixture was stirred for 48 hours at 20°C. The polymerization medium was concentrated by cryo-distillation and the copolymer was recovered by precipitation into cold diethyl ether. The polymer was recovered as a white powder after three washings with diethyl ether and drying under dynamic vacuum for 24 hours. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): δ = 7.17-7.39 [458H, Ar-H\textsubscript{F}], 4.97-5.19 [180H, CH\textsubscript{E}], 4.49-4.7 [89H, CH\textsubscript{B}], 3.64-3.88 [453H, PEO backbone CH\textsubscript{G}], 3.5 [3H, OCH\textsubscript{H}], 2.3-2.7 [190H, CH\textsubscript{D}], 1.79-2.2 [201H, CH\textsubscript{C}].

The degree of polymerization of PBLG measured by proton NMR was DP = 90, corresponding to a molar mass of 25.9 kg/mol. Molar mass dispersity was obtained by SEC in DMF (LiBr, 60 °C): D = 1.18. An α-helical structure was evidenced by circular dichroism showing characteristic dips at 208 and 222 nm (Fig. S6).\textsuperscript{S3}

PEG-b-PBLG was modified in bulk using succinimidyl 3-(2-pyridyldithiolpropionate) (SPDP, Pierce). Briefly, PEG-b-PBLG copolymer (1 g, ~40 μmol) was dissolved in anhydrous DMF (0.1 g/mL) and N,N-diisopropylethylamine (DIPEA) (6μL; 1 eq. per amine function) was added. After 5 min stirring, SPDP (20 mg; 1.7 eq.) was added and the mixture was stirred...
overnight at room temperature under N$_2$ atmosphere. The resulting product was recovered by precipitation in cold diethyl ether and dried under vacuum.

The theoretical length of the PEG-$b$-PBLG was estimated and used as an additional parameter to prove the probing of individual molecules during the pulling experiments. An $\alpha$-helix is characterized by a translation of 0.15 nm per amino acid in a folded state, whereas this distance rises to 0.37 nm in an extended form (without angles and bonds deformation). The length increment per amino acid is therefore 0.22 nm, leading to a maximum length increase of ~20 nm (= 90 x 0.22 nm). The maximum extended length of PEG is ~40 nm (= 113 x 0.35 nm). Thus, the extended length of the molecule before and after the $\alpha$-helix unfolding is 55 (= 40 + 0.15 x 90) and 75 (= 40 + 0.37 x 90) nm respectively.

2. Substrate cleaning and molecules immobilization

Gold-coated silicon substrates were prepared using our previously established protocol. Surfaces were cut and cleaned under UV/ozone (UV-ozone cleaner®, Model 42, Jelight Company Inc), followed by a dipping time of 20 min in pure ethanol to reduce the gold oxides formed at the surface and thus favor the interaction with sulfur compounds. The grafting of PEG-$b$-PBLG onto the surface is performed in the presence of an additional small molecule, PEG$_6$-SH, used as a passivation agent that disperses the molecules of interest on the surface and decreases unspecific tip-surface adhesion during force experiments. We prepared a grafting solution with a molar ratio of 85/15 ([PEG$_6$-SH]/[PEG-$b$-PBLG]) and [PEG-$b$-PBLG] = 0.1 g/L in dioxane. The Au/Si surfaces were dipped in the grafting solution for 15 min, leading to a substrate with low grafting density favoring single-molecule experiments. Subsequent washing with fresh dioxane provides the final surface directly used for AFM experiments.
3. AFM-based SMFS experiments

AFM experiments were performed using a PicoPlus 5500 microscope (Agilent Technologies) equipped with a closed-loop scanner. MSCT tips (Bruker, Si₃N₄) with nominal spring constant of $k = 0.03 \text{ N} \cdot \text{m}^{-1}$ were used in standard force experiments, whereas softer tips with $k = 0.004 \text{ N} \cdot \text{m}^{-1}$ were used for pulling-relaxing experiments. The spring constant of each cantilever was calibrated in air using the thermal noise method implemented in the AFM software. Before each experiment, a new AFM chip was cleaned by UV/ozone (15 min). The grafted surface was installed in a closed fluid cell filled with fresh dioxane, the AFM tip was immersed in the solution away from the surface for 1h for equilibration of the cantilever. In case of experiments in denaturing environment, a solution of dioxane and trifluoroacetic acid (80/20 v:v) was prepared and used as solvent in the closed fluid cell.

The molecules were gently picked up using a maximum force of 1 nN against the substrate to promote the physisorption of PEG onto the AFM tip (no dwell time at contact). Standard force curves were obtained at a fixed velocity of 200 nm·s⁻¹ (approach and retraction), whereas pulling-relaxing experiments were performed at 50 nm·s⁻¹. Force-extension curves were obtained by transforming the deflection-piezo movement curves using the Hooke’s law:

$$ F = k \Delta x \quad \text{and} \quad d = Z - \Delta x $$

with $F$ the force experienced by the molecule, $k$ the spring constant of the cantilever, $\Delta x$ the cantilever deflection, $z$ the piezo-movement and $d$ the tip-substrate distance.

In pulling–relaxing experiments—before and after every cycle—a few curves in which no molecule has been stretched have been selected as references. From the comparison between these measurements possible drift could be identified. The baseline of the curve before the cycle is used as the zero force value for the first curve of the cycle; the stretching profiles of the
successive curves are superimposed and the consistency of the proposed zero force value is
tested on the last curve of the cycle, when the molecule has been lost and the force drops to
zero. The zero extension is the reference position of the piezo (contact point) as shown by the
change in the slope of the force extension curve which becomes vertical. The procedure to
identify the zero extension during the cycle is analogous to the one used to identify the zero
force. The zero length position identified in the last curve before the cycle is assigned to the
first curve of the cycle, while the stretching profiles of the curves of the cycle are superimposed
and the consistency of the proposed position of the zero length is tested in the first curve after
the cycle.

4. Data analysis
The raw deflection-piezo movement curves were sorted out using a home-made routine on
IgorPro (WaveMetrics) to discriminate characteristic unfolding patterns from unspecific
profiles. More than 95% of the curves show flat profiles, indicating that no molecule was
stretched during the approach-retraction cycle. This observation is typical of an experiment
performed in highly diluted grafting conditions, favoring single-molecule attachment.
Following the transformation to force-distance curves (described before), analysis routines
were used to determine the final tip-molecule rupture force and extension, the plateau length
and the plateau force associated to each characteristic unfolding pattern. The mean force of
each plateau was determined using a linear fit over the whole plateau with a slope fixed at zero
to avoid any bias originating from the noise. Pulling-relaxing curves were smoothed using
IgorPro for more clarity (raw data is shown as lighter dots when a smoothing process was
performed).
Histograms of $D_R$, $F_R$, $\Delta L$ and $F_P$ were constructed using IgorPro (WaveMetrics). Raw data were fitted using a Gaussian mixture model (GMM) on MatLab (MathWorks), i.e., a weighted sum of M components ($i = 1, 2, 3$) Gaussian densities as given by:

$$P(x|\lambda) = \sum_{i=1}^{M} p_i G(x | \mu_i, \sigma_i)$$

where $x$ is the vector of the observable, $G(x | \mu_i, \sigma_i)$ is the normalized Gaussian component with mean $\mu_i$ and variance $\sigma_i$, and $p_i$ is the weight of the $i^{th}$ component. The weights satisfy the normalization condition $\sum_{i=1}^{M} p_i = 1$, and $\lambda$ represents the set of all the parameters $\lambda = \{p_i, \mu_i, \sigma_i\}$ for $i=1,2$ or 3.

Each population is given with its 95% confidence interval estimated as $\pm 1.96 \frac{\sigma^2}{(p_i N)}$ where $\sigma^2$ is the estimated variance of the $i^{th}$ component while $p_i N$ represents the effective size of the population. Probability density function (PDF) were also obtained by fitting the data by a Kernel smoothing function ($N = 1000$ points) on MatLab (MathWorks).

5. Supplementary figures
Fig. S1 Distributions of plateau length and plateau force in dioxane in the case of stretching via PBLG, i.e. associated with $D_R \leq 35$ nm. (a) Distribution of plateau length ($\Delta L$) shows two populations centered at $9.3 \pm 0.4$ nm (76%) and $14.0 \pm 0.6$ nm (24%), corresponding to the unfolding of helices made of $42 \pm 2$ AA and $64 \pm 3$ AA respectively. The unfolding of fewer helices is in agreement with the attachment of the tip along the PBLG chain, reducing the number of intact helices available between the tip and the surface during stretching. (b) Distribution of plateau force ($F_P$) showing one population centered at $47.9 \pm 2.6$ pN.
Fig. S2 Distributions of plateau length and plateau force in dioxane in the case of stretching via the PEG tether, i.e. associated with $D_R > 35$ nm. (a) Distribution of plateau length ($\Delta L$) shows three populations centered at $9.7 \pm 0.2$ nm (17%), $13.1 \pm 0.6$ nm (72%) and $19.0 \pm 0.7$ nm (11%), corresponding to the unfolding of helices made of $44 \pm 1$ AA, $60 \pm 3$ AA and $87 \pm 3$ AA respectively. The unfolding of a higher number of helices is in agreement with the attachment of the tip along the PEG tether. (b) Distribution of plateau force ($F_P$) presents one population centered at $46.1 \pm 1.5$ pN, very close to the value in the case of the pulling via PBLG, evidencing the rupture of similar interactions.
Fig. S3 Proportions of a single α-helix (about 20 AA), 2 helices (40 AA), 3 helices (60 AA) and 4 helices (80 AA) observed during the stretching of the PBLG-PEG, depending on the tip attachment point. (Left) Experiments in dioxane with $D_R < 35$ nm (attachment on PBLG); (right) experiments in dioxane with $D_R > 35$ nm (attachment on PEG). As expected, attaching the molecule by the PBLG or the PEG chain influences the number of intact α-helices probed.
**Fig. S4** Distributions of plateau length and plateau force for the stretching of PEG-PBLG in dioxane-TFA. (a) Distribution of the plateau length ($\Delta L$) shows three populations centered at 3.5 ± 0.1 nm (8%), 9.6 ± 0.8 nm (58%) and 14.4 ± 0.6 nm (34%) corresponding to the unfolding of helices made of 16 ± 1 AA, 44 ± 4 AA and 65 ± 3 AA respectively. Probability density function (PDF) fit is added in dotted line. The decrease of the number of helices in series and the apparition of a population of about 20 AA corresponding to a single helix is consistent with the presence of a denaturating agent (TFA). (b) Distribution of the plateau force ($F_P$) in dioxane-TFA presents one population centered at 37.4 ± 3.8 pN, lower than $F_P$ observed in dioxane, again evidencing the destabilizing effect of TFA.
Fig. S5 Proportions of a single $\alpha$-helix (about 20 AA), 2 helices (40 AA), 3 helices (60 AA) and 4 helices (80 AA) observed during the stretching of the PBLG-PEG in dioxane (left) and in TFA (right). As expected, the number of $\alpha$-helical content is decreasing in presence of TFA (denaturing agent). Single $\alpha$-helices of about 20 residues are only observed in denaturing conditions during standard pulling experiments.

Fig. S6 Circular dichroism spectrum of the PBLG in dioxane showing characteristic dips of a $\alpha$-helical structure (208 and 222 nm).
6. References


