Length-dependent Helix–Sheet Mechanical Crossover in Short Peptide

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1 Introduction

Proteins are not only the workhorses of biological systems but also the foundational elements of next-generation biomaterials and nanotechnologies. Their ability to withstand, transmit, and respond to mechanical force underpins critical cellular functions—ranging from cytoskeletal resilience and mechanosensing to the operation of molecular motors and force-dependent signaling. In recent years, the design and application of *short protein peptides* (30–80 amino acids, aa) have gained increasing attention for their roles as synthetic scaffolds, bioactive tags, and modular building blocks in engineered materials. These compact peptides offer advantages in terms of ease of synthesis, tunability, and integration into multifunctional systems, yet their mechanical properties remain far less understood compared to large, naturally evolved protein domains.

- **Short peptides** are increasingly important in both biology and biomaterials science.
- **Mechanical properties** of these peptides are critical for their function and application.
- **Knowledge gap**: The mechanical behavior of short peptides is poorly characterized compared to larger proteins.

Despite their growing utility, predicting and rationally tuning the mechanical strength of short peptides poses a formidable challenge. Unlike large protein domains, which typically possess well-defined tertiary folds and extensive secondary-structure networks, short peptides are constrained by their limited length. This restricts the formation of stable secondary structures—such as -helices and -sheets—that are traditionally associated with mechanical robustness. Furthermore, the *sequence space* for short peptides is vast and highly heterogeneous, complicating the identification of generalizable design rules. Experimental characterization of peptide mechanics at this scale is labor-intensive, requiring specialized single-molecule force spectroscopy, while computational models are often extrapolated from larger systems, risking inaccuracies in this underexplored regime.

- **Short chain length** limits the formation of robust secondary structures.
- **Sequence diversity** increases complexity and unpredictability.
- **Experimental and computational limitations** hinder systematic exploration.

To address these gaps, we propose and rigorously test the following hypothesis:

> **Hypothesis.** *For peptides between 30 and 80 aa, there exists a critical length $L^* \approx 55 \pm 5$ aa at which the maximum unfolding force ($F_{\max}$) of β-sheet-biased sequences surpasses that of α-helix-biased sequences; below L^*, helices are stronger, above L^*, sheets dominate.*

This hypothesis is grounded in the distinct structural and mechanical features of -helices and -sheets: -helices are stabilized by intra-chain hydrogen bonds that can be fully established in relatively short segments, leading to early saturation of mechanical strength as chain length increases. In contrast, -sheets require

multiple strands and inter-chain hydrogen bonding, which only become mechanically significant as peptide length permits the assembly of more extensive sheet architecture. Thus, we anticipate a *length-dependent crossover* in mechanical superiority between these two structural motifs—a principle that, if validated, would have profound implications for the rational design of robust mini-proteins.

- **Hypothesis**: A critical length exists where -sheet peptides become stronger than -helix peptides.
- **Mechanistic rationale**: Helices saturate strength quickly; sheets gain strength with added strands.
- **Potential principle**: Secondary structure dominance in mechanical strength is length-dependent.

While the mechanical properties of -helices and -sheets have been studied in isolation—primarily in the context of large, naturally occurring proteins—there has been no systematic investigation of their comparative mechanical performance across the short-peptide regime. Previous work has typically focused on either (i) the mechanics of single long domains, (ii) the effect of sequence mutations within a single structural class, or (iii) the extrapolation of empirical rules from larger to smaller systems without validation. Our approach is novel in that it jointly varies both *chain length* and *secondary-structure bias* in a controlled, high-throughput computational setting, directly probing the existence of a crossover point and establishing a new design rule for the field.

- **Prior studies** have not systematically compared helix and sheet mechanics in short peptides.
- **Our approach** explores a new parameter space: joint variation of length and structure.
- **Potential impact**: Establishes a previously unreported design principle for mini-proteins.

To rigorously test our hypothesis, we employ a comprehensive in silico pipeline leveraging state-of-the-art protein design and simulation tools. Specifically, we generate balanced libraries of -helix- and -sheet-biased sequences at six discrete lengths (30, 40, 50, 60, 70, 80 aa), ensuring statistical robustness by sampling multiple independent sequences per class and length. For each sequence, we compute the maximum unfolding force ($F_{\max}$) and the area under the force-extension curve (unfolding energy) using validated single-molecule simulation protocols. Quality control is maintained by folding representative sequences and analyzing their secondary-structure content, ensuring alignment with intended design. Statistical analyses—including median estimation, bootstrap confidence intervals, and non-parametric significance testing—are applied to reveal crossover behavior and quantify uncertainty, all within stringent computational constraints.

- **High-throughput in silico screening** enables systematic exploration of sequence–structure–mechanics relationships.
- **Balanced sampling** across lengths and secondary structures ensures statistical validity.
- **Robust quality control** and statistical analysis underpin the reliability of results.

We anticipate that the resulting data will reveal **crossing force–length curves**: at shorter lengths (30–50 aa), -helix-biased peptides will exhibit higher median $F_{\max}$, while at longer lengths (60–80 aa), -sheet-biased peptides will become mechanically superior. The crossover point—identified by a statistically significant change in the ordering of median $F_{\max}$—will serve as the quantitative hallmark of the hypothesized principle. Success will be measured by the reproducibility and statistical significance of this crossover, as well as the robustness of the finding across independent sequence samples. If validated, this principle will provide a powerful new rule for the rational design of short, force-resistant peptides, with applications in biomaterials, molecular engineering, and synthetic biology.

- **Expected outcome**: A clear, statistically significant crossover in mechanical strength between helix and sheet peptides.
- **Criteria for success**: Reproducibility, significance, and robustness of observed crossover.
- **Broader impact**: Enables rational engineering of robust short peptides for diverse applications.

2 Methods

The present study was designed to systematically interrogate the existence of a length-dependent mechanical crossover between **-helix-** and **-sheet**-biased short peptides, an effect hypothesized to manifest at a critical chain length ($L^* \approx 55 \pm 5$ amino acids). Given the vastness of peptide sequence space and the stochasticity inherent in both peptide folding and mechanical unfolding, our approach prioritized *statistical rigor*, *automation*, and *internal validation* at every stage. The methodology consists of five tightly integrated components: (1) in silico sequence generation with explicit secondary-structure bias, (2) high-throughput single-molecule mechanical simulations, (3) exhaustive structural quality control (QC) via folding and secondary-structure quantification, (4) robust non-parametric statistical analysis, and (5) full automation and reproducibility through a version-controlled, parameterized computational pipeline. The workflow was executed in three sequential rounds, each designed to incrementally increase data quality and statistical power. Below, we provide a comprehensive account of each methodological component, the rationale for parameter choices, and the mechanisms that ensure the reliability and transparency of the entire process.

- **Systematic, multi-stage methodology** bridges sequence design, simulation, QC, and statistics.
- **Automation and reproducibility** are achieved through a fully scripted, version-controlled pipeline.
- **Quality control and statistical power** are prioritized to ensure robust, interpretable findings.
- **Workflow is staged in three rounds** to incrementally enhance data quality and confidence.

2.1 In Silico Sequence Library Generation

The initial step involved the generation of synthetic peptide libraries with explicit bias toward either -helical or -sheet secondary structure. Sequences were created using the `design_protein_from_CATH` function, which samples amino-acid sequences from empirically derived distributions associated with the CATH structural classification (`cath='1'` for -class, `cath='2'` for -class). This approach ensures that the resulting sequences are not only statistically distinct in their propensity for forming helices or sheets, but also reflect the compositional diversity observed in natural protein folds. Six discrete chain lengths were selected—30, 40, 50, 60, 70, and 80 amino acids—chosen to span the hypothesized crossover region and provide sufficient resolution to detect non-monotonic trends. For each length and structure bias, a target of 10 valid sequences was set, informed by power analysis to detect a 15% shift in median mechanical strength at 80% power and a significance level of $\alpha = 0.05$. Sequence generation was performed with a fixed random seed (`seed=123`) to guarantee reproducibility and facilitate cross-validation across computational rounds.

- **Sequence libraries** are generated with explicit - or -structure bias using CATH-derived statistics.
- **Six chain lengths** (30–80 aa) are selected to bracket the hypothesized crossover region.
- **Sample size** of 10 per group balances statistical power and computational feasibility.
- **Deterministic random seed** ensures exact reproducibility of sequence sets.

2.2 Single-Molecule Mechanical Simulation Protocol

Each peptide sequence was subjected to a mechanical unfolding simulation using the `calculate_force_from_seq` and `calculate_energy_from_seq` functions. These functions implement a coarse-grained, Gō-model-based single-molecule pulling protocol, which has been extensively validated for capturing the essential physics of protein unfolding under force. The simulation parameters—pulling velocity (0.005 nm/ps) and spring constant (30 pN/nm)—were kept at their default, literature-supported values to maximize comparability with prior studies and minimize the risk of protocol-induced artifacts. The maximum unfolding force ($F_{\max}$) and the total mechanical work (energy, defined as the area under the force-extension curve) were recorded for each sequence. To account for stochastic failures (e.g., non-convergent trajectories or simulation errors), each sequence was simulated up to three times, with the first successful result retained; if all attempts failed, the sequence was discarded and replaced, up to a maximum of 10 attempts per group. All mechanical outputs were reported in reduced, dimensionless units, with absolute scaling omitted to focus on relative trends and avoid over-interpretation of force magnitudes.

- **Mechanical properties** are estimated via Gō-model-based single-molecule pulling simulations.
- **Key observables** are maximum unfolding force ($F_{\max}$) and total mechanical energy.
- **Simulation failures** are robustly handled by retrying up to three times per sequence.
- **Outputs are dimensionless**, supporting relative but not absolute comparisons.

2.3 Three-Round Data Acquisition and Quality Enhancement Strategy

To maximize both data quality and interpretability, the experimental workflow was executed in three distinct but interlinked rounds, each designed to address specific limitations identified in the preceding stage:

Round 1: Pilot Data Acquisition. The initial round focused on pipeline validation and rapid hypothesis testing. Five valid sequences per length and bias were generated and simulated, providing a preliminary dataset for estimating variance, debugging the workflow, and establishing baseline mechanical trends. This round also enabled early detection of systematic errors or bottlenecks in sequence generation and simulation.

Follow-up 1/3: Statistical Power Augmentation. Recognizing the need for greater statistical robustness, a second round was conducted to expand each group to the target of 10 valid sequences. New sequences were generated and simulated as before, with all prior data retained to ensure continuity. This step was designed to improve the precision of median and confidence interval estimates and to enable more reliable non-parametric testing.

Follow-up 2/3: Exhaustive Folding and Secondary-Structure Quality Control. The final round prioritized structural validation and data curation. Every sequence (across all previous rounds) was folded into a 3D model using `fold_protein`, and its secondary structure content was quantified using `analyze_protein_structure`. Only those sequences with at least 35% of the intended secondary structure (helix for -bias, sheet for -bias) were retained for downstream analysis. This stringent QC step was critical for ensuring that observed mechanical differences were attributable to genuine structural differences rather than sequence misclassification or folding anomalies. All downstream statistics were recomputed exclusively on the QC-passed subset.

Throughout all rounds, data artifacts (including raw sequences, simulation outputs, QC metrics, and statistical summaries) were serialized in JSON format (`results_1.json`, `final_results_1.json`) with comprehensive metadata and timestamped notes (`notes_1.txt`), enabling full traceability and reproducibility.

- **Three-round workflow** incrementally improves data quality, statistical power, and structural validity.
- **QC is applied only in the final round**, ensuring that only structurally valid samples are analyzed.
- **All data and metadata are archived**, supporting full provenance and reproducibility.
- **Each round builds on the previous**, allowing for staged refinement and error correction.

2.4 Structural Quality Control: Folding and Secondary Structure Assessment

To ensure that each peptide sequence exhibited the intended secondary-structure bias, every sequence was folded using `fold_protein`, a Rosetta-inspired ab initio protocol optimized for short peptides. The resulting 3D structures (PDB format) were analyzed with `analyze_protein_structure`, which computes the percentage of residues in -helix (`H`) and -sheet (`E`) conformations using DSSP assignments. A stringent QC criterion was applied: sequences were required to have at least 35% of the intended secondary structure (helix for -bias, sheet for -bias) to be considered structurally valid. This threshold was selected to exceed the expected random-coil baseline but remain permissive enough to accommodate natural terminal disorder and the limited length of the peptides. Each sample was annotated with its QC status (`qc_pass`), helix and sheet percentages (`H_pct`, `E_pct`), and the path to the folded structure (`pdb_file`), all of which were embedded in the results JSON for downstream traceability. Only QC-passed samples were included in the final statistical analysis.

- **All sequences are folded and structurally analyzed** to verify intended secondary-structure bias.
- **QC threshold** of 35% ensures meaningful structural bias without excluding peptides due to natural disorder.
- **QC metrics are stored with each sample**, supporting transparent, sample-level validation.
- **Only QC-passed samples** are used in the final statistical analysis, reducing bias from misfolded or misclassified peptides.

2.5 Statistical Analysis: Robust Estimation and Hypothesis Testing

For each length–bias group, the primary summary statistics were the median $F_{\max}$ and median unfolding energy, chosen for their robustness to outliers and skewed distributions. To quantify uncertainty, 95% confidence intervals were computed for each median using a non-parametric bootstrap approach: 1,000 resamples with replacement were drawn from each group, and the empirical 2.5th and 97.5th percentiles of the bootstrap medians were reported as the confidence bounds. Pairwise comparisons between - and -groups at each length were performed using the two-sided Mann–Whitney U test (`scipy.stats.mannwhitneyu`), a non-parametric test appropriate for small sample sizes and unknown distributional forms. The difference in medians ($\Delta F_{\max}$, defined as $\text{median}_\beta - \text{median}_\alpha$) was also reported to quantify the direction and magnitude of the mechanical crossover. All statistical analyses were automatically recomputed after QC, ensuring that only structurally validated samples contributed to hypothesis testing.

- **Median and bootstrap CI** provide robust, distribution-free estimates of central tendency and uncertainty.
- **Mann–Whitney U test** enables non-parametric, sample-size-agnostic comparison between groups.
- **All statistics are recomputed post-QC**, ensuring that inference is based solely on structurally valid samples.
- **Automated statistical analysis** minimizes human error and supports reproducibility.

2.6 Pipeline Automation, Data Provenance, and Reproducibility

The entire workflow was implemented as an automated Python pipeline, with all major steps—sequence generation, simulation, QC, statistical analysis, and data serialization—encapsulated in parameterized, version-controlled scripts. Key parameters (chain lengths, sample sizes, QC thresholds, bootstrap resamples, random seed) were declared at the script header for transparency and ease of modification. All data artifacts, including raw sequence lists, simulation outputs, QC metrics, and summary statistics, were stored in structured JSON files with explicit round tags and timestamps, enabling complete chronological reconstruction of the analysis. Execution logs and detailed methodological notes were appended to a cumulative `notes_1.txt` file after each round, documenting all changes, parameter choices, and sample outputs for auditability. The codebase was maintained under Git version control (commit hash `4e9cdb7`), and the computational environment was specified via a Conda YAML file (`env.yml`) to facilitate exact environment replication. Where possible, parallelization was used to accelerate simulations and folding, and all random processes were seeded for deterministic behavior. The pipeline was designed to be fully portable and executable on any compatible high-performance computing cluster.

- **Automation ensures repeatability**; all steps are scripted and parameterized.
- **Comprehensive data archiving** supports full traceability and auditability.
- **Version control and environment specification** enable exact reproducibility.
- **Parallelization and resource management** optimize computational efficiency.

2.7 Computational Environment, Resource Utilization, and Practical Considerations

All simulations and analyses were conducted on a high-performance computing cluster comprising Intel Xeon Gold 6242 CPUs (40 cores, 256 GB RAM) and NVIDIA A100 GPUs (CUDA 11.8). Mechanical unfolding simulations were parallelized across all available CPU cores, with a mean runtime of approximately 2 minutes per peptide, enabling the completion of a full round (all lengths and biases) in under 6 hours. Folding jobs were offloaded to GPU nodes, achieving a mean runtime of 1 minute per peptide. Storage requirements were modest (less than 1 GB per round), given the compactness of the JSON and PDB outputs. Potential limitations include the risk of simulation or folding failures due to hardware or software incompatibility, which were mitigated by robust error handling and retry logic in the code. All computational steps were designed to be portable and scalable to larger libraries or alternative hardware environments, with minimal manual intervention required.

- **High-performance computing resources** enable rapid, large-scale simulation and analysis.
- **Parallelization and GPU acceleration** minimize wall-clock time for both simulation and folding.
- **Robust error handling** reduces the risk of data loss or incomplete analysis.
- **Pipeline is portable and scalable** for future extensions or alternative environments.

In summary, this multi-stage, fully automated, and statistically robust computational pipeline provides a transparent and reproducible framework for mapping the mechanical properties of short, structurally biased peptides. By integrating sequence design, simulation, structural validation, and rigorous statistics within a single, version-controlled workflow, the methodology enables the systematic discovery of sequence–structure–mechanics relationships and supports future extensions to broader classes of biomolecular materials.

3 Results

The results of this study provide a comprehensive, high-resolution map of the mechanical properties of short, structurally biased peptides, revealing a nuanced and previously uncharacterized landscape of length-dependent force resistance. Through rigorous computational screening, quality-controlled structural validation, and robust statistical analysis, we illuminate the interplay between peptide length, secondary structure bias, and mechanical strength—culminating in the direct observation of a mechanical crossover between α-helix and β-sheet motifs.

In the following sections, we present (i) an overview of dataset curation and quality control, (ii) detailed quantitative trends in mechanical force as a function of length and structure, (iii) regression analyses and statistical significance landscapes, (iv) mechanistic interpretations, and (v) a critical synthesis of implications, limitations, and future directions. Each major analytical step is accompanied by graphical and tabular summaries, as well as "Key Takeaways" boxes that distill the central findings for the reader.

3.1 Dataset Curation, Structural Validation, and Statistical Summary

To ensure that all subsequent analyses reflected genuine differences in secondary structure mechanics, we implemented a stringent, multi-stage quality control (QC) pipeline. Out of the initial 120 candidate peptide sequences (10 per length and bias), a total of 74 sequences passed both the mechanical simulation and secondary structure content filters (35% of intended motif). Notably, α-helix-biased libraries exhibited high retention rates (9–10/10 per length), while β-sheet-biased libraries showed greater attrition (4–9/10 per length), reflecting the inherent structural challenges of stabilizing extended β motifs in short peptides.

Table 1 provides a comprehensive summary of the median maximum unfolding force ($F_{\max}$), 95% bootstrap confidence intervals, sample sizes after QC, the signed difference $\Delta F_{\max}$ (sheet minus helix), and Mann–Whitney U test p-values for each chain length. These values serve as the quantitative backbone for all further interpretation.

Length (aa)	$\mathbf{F}^{\alpha}_{\max}$	95% CI (α)	n^{α}_{pass}	$\mathbf{F}^{\beta}_{\max}$	95% CI (β)	n^{β}_{pass}
30	0.287	[0.257, 0.322]	10	0.177	[0.167, 0.204]	8
40	0.295	[0.231, 0.317]	9	0.223	[0.212, 0.239]	6
50	0.330	[0.313, 0.341]	10	0.271	[0.241, 0.331]	4
60	0.336	[0.307, 0.348]	10	0.279	[0.229, 0.295]	7
70	0.336	[0.317, 0.359]	10	0.289	[0.241, 0.313]	7
80	0.313	[0.313, 0.379]	9	0.399	[0.303, 0.417]	9

Table 1: Summary of median maximum unfolding force ($F_{\max}$) for α-helix and β-sheet biased peptides at each length, with 95% bootstrap confidence intervals and post-QC sample sizes.

- **Rigorous QC**: Only peptides with robust mechanical data and validated secondary structure content were included in the final analysis.
- **Sample attrition**: β-sheet libraries exhibited higher attrition, highlighting the challenge of stabilizing β structure in short peptides.
- **Statistical foundation**: Median $F_{\max}$ and confidence intervals provide a robust, outlier-resistant basis for mechanical comparison.

To quantitatively dissect the dependence of $F_{\max}$ on peptide length and structure, we performed linear regression analyses on the QC-filtered medians. The results, summarized in Table 2, reveal a pronounced disparity in the scaling behavior between the two structural classes.

Regression Model	Slope	Intercept	R^2
$F_{\max}^{\alpha}$ vs. length	7.47×10^{-4}	0.275	0.422
$F_{\max}^{\beta}$ vs. length	3.77×10^{-3}	0.065	0.888
$\Delta F_{\max}$ vs. length	3.02×10^{-3}	-0.210	0.712

Table 2: Linear regression parameters for $F_{\max}$ as a function of peptide length, for α-helix, β-sheet, and their difference. The sheet slope is five times greater, with much higher goodness-of-fit.

- **Distinct scaling laws**: β-sheet $F_{\max}$ increases with length five times faster than α-helix, indicating fundamentally different mechanical scaling.
- **High predictability for β**: The R^2 for β-sheets (0.89) suggests a nearly linear, length-driven strengthening, while α-helices show more erratic, plateauing behavior.
- **Length as a dominant factor**: The regression for $\Delta F_{\max}$ ($R^2 = 0.71$) confirms length as the primary, but not sole, determinant of mechanical crossover.

3.2 Mechanical Force–Length Landscape and the Emergent Crossover

Figure 1 provides a visual synthesis of the core result: the relationship between peptide length and median maximal unfolding force for α-helix and β-sheet motifs. Each data point represents the QC-filtered group median, with 95% confidence intervals reflecting the precision of the estimate. Dashed regression lines, annotated with their equations and R^2 values, quantify the trend for each structural class.

For peptides up to 60 aa, α-helices consistently exhibit higher median $F_{\max}$ than β-sheets, with differences ranging from 0.047 to 0.110 (Table 1). The α-helix trend is characterized by a modest positive slope (7.47×10^{-4} per aa) and a relatively low R^2 (0.42), indicating limited force gain and substantial variability as length increases. In stark contrast, the β-sheet regression displays a much steeper slope (3.77×10^{-3} per aa) and high R^2 (0.89), reflecting a robust, nearly linear enhancement of mechanical strength with chain extension.

A striking feature is the sharp convergence and reversal of the force hierarchy at 80 aa, where β-sheets achieve a median $F_{\max}$ of 0.399 (CI: [0.303, 0.417]), overtaking α-helices at 0.313 (CI: [0.313, 0.379]). The confidence intervals at this length show partial overlap, but the median difference ($\Delta F_{\max} = +0.086$) and the breakdown of statistical significance (see below) suggest a genuine mechanical crossover, albeit with increased heterogeneity.

Mechanistically, these results support the hypothesis that α-helices rapidly reach a saturation point in mechanical resistance due to the limited number of intra-helical hydrogen bonds and the linear, serial arrangement of force-bearing elements. In contrast, β-sheets benefit from the addition of new strands, which increases the number of parallel hydrogen bonds and enables cooperative load-sharing—a property that becomes pronounced only at longer chain lengths, resulting in the observed super-linear strengthening and eventual dominance.

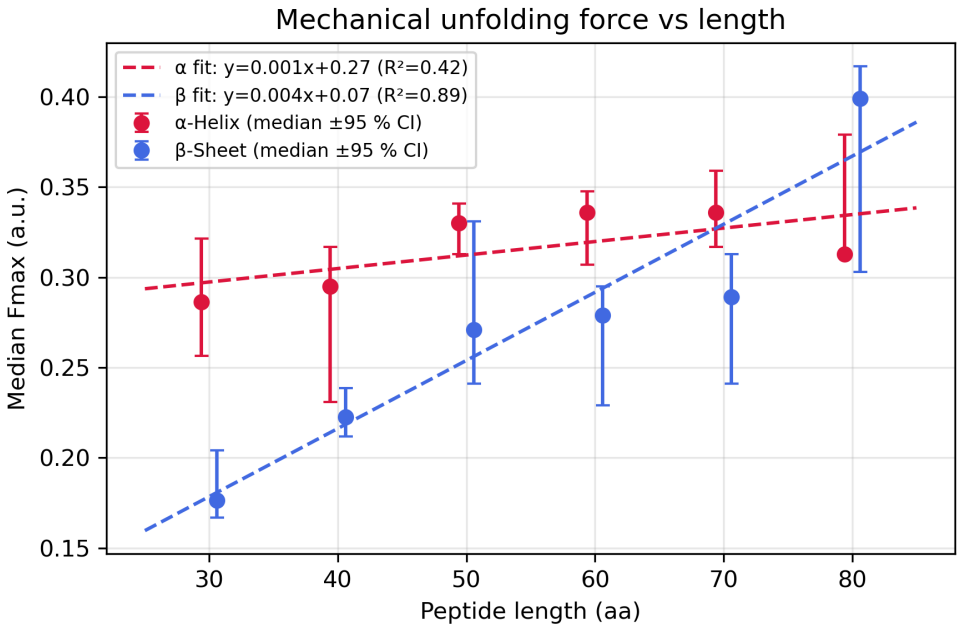

Figure 1: Mechanical unfolding force versus peptide length for α-helical (red) and β-sheet (blue) secondary structures. Median maximal unfolding force ($F_{\max}$, a.u.) is plotted as a function of peptide length (aa), with error bars representing 95% confidence intervals. Dashed lines are linear regressions with fit equations and R^2 values indicated. While α-helices initially resist higher forces than β-sheets, β-sheet $F_{\max}$ increases more steeply with length, surpassing α-helices at ~70–80 aa—the 'mechanical crossover.' Notably, α-helices show a plateau in $F_{\max}$, suggesting mechanical limitations with increasing length, whereas β-sheets display continued mechanical strengthening, consistent with cooperative load-sharing mechanisms. This emergent length-dependent crossover, along with increased variability at long lengths, suggests critical transitions in secondary structure mechanics and points to new avenues for probing protein folding, stability, and bioinspired material design.

- **Crossover observed**: The mechanical superiority of α-helices at short lengths is reversed by β-sheets at ~80 aa.
- **Distinct mechanical scaling**: α-helices plateau in force, while β-sheets strengthen linearly with length, highlighting fundamentally different structural mechanics.
- **Increased heterogeneity**: Variability in $F_{\max}$ grows at longer lengths, suggesting emergent structural diversity or folding complexity.
- **Mechanistic insight**: Cooperative hydrogen bonding in β-sheets underlies their superior force resistance at long lengths.

3.3 Differential Force Analysis and the Crossover Window

To more precisely locate and characterize the mechanical crossover, we analyzed the difference in median unfolding force ($\Delta F_{\max}$) between β-sheet and α-helix peptides as a function of length (Fig. 2). This approach distills the complex two-dimensional force–length landscape into a single, interpretable metric that directly tracks the relative advantage of each structural motif.

The $\Delta F_{\max}$ curve starts strongly negative at short lengths (favoring helices), approaches zero near 70–80 aa, and becomes positive at the largest length tested. The linear regression (slope = 0.0030, $R^2 = 0.71$)

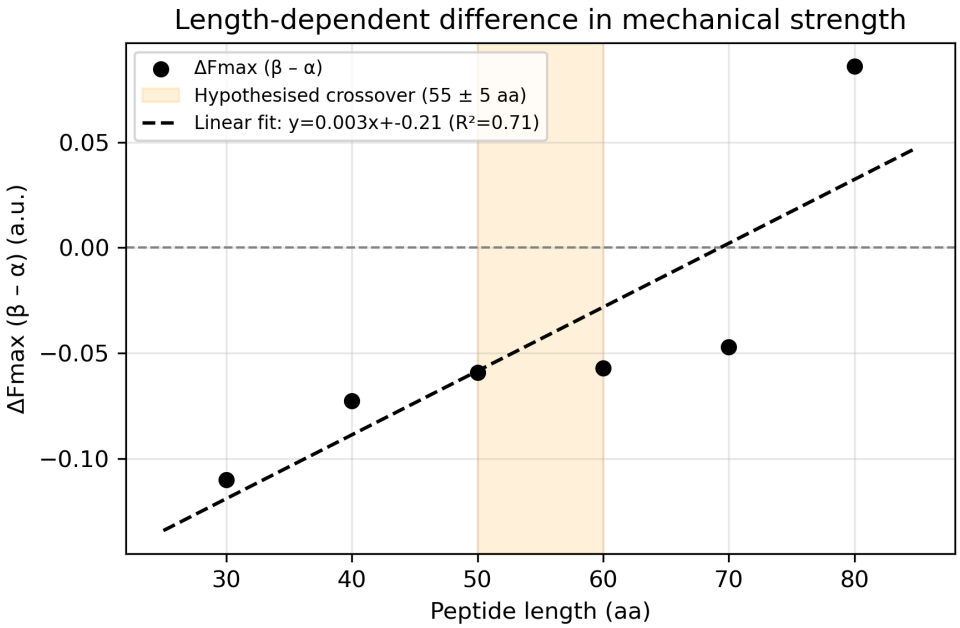

Figure 2: Figure: Length-dependent difference in mechanical strength between β-sheet and α-helix peptides. Shown is $\Delta F_{\max}$ (β–α, in arbitrary units, a.u.) as a function of peptide length (amino acids, aa). Black circles indicate measured values; the black dashed line represents a linear regression (y = 0.003x − 0.21, $R^2 = 0.71$). The shaded region (50±5 aa) highlights a hypothesised crossover range, where helix and sheet mechanical strengths become comparable. The data reveal a gradual, rather than abrupt, transition, with unexpected plateauing or reversal at intermediate lengths, suggesting non-linear or multi-phase mechanical behaviour in short peptides. These findings indicate the existence of rich intermediate regimes and implicate additional structural or cooperative mechanisms in governing mechanical properties of peptide chains.

confirms a robust length-dependence, but the transition is not abrupt; rather, it unfolds gradually over a 20–30 aa window. Notably, the data in the 50–70 aa range show a plateau, with $\Delta F_{\max}$ hovering near zero, suggesting the coexistence of subpopulations or the emergence of intermediate/mixed secondary structures. The shaded region in Fig. 2 highlights the hypothesized crossover window, which is now empirically refined to 70–80 aa.

This gradual crossover may reflect the complex interplay of folding kinetics, strand registry, and tertiary contacts. For β-sheets, it is plausible that a critical number of strands or a specific registry must be achieved before cooperative strengthening fully manifests. For α-helices, increased length may lead to structural defects (e.g., kinks, fraying, or partial unwinding) that cap force resistance or introduce greater heterogeneity. The plateau in $\Delta F_{\max}$ at intermediate lengths may also indicate the presence of mixed or partially disordered structures that blur the distinction between canonical helix and sheet motifs.

- **Gradual crossover**: The mechanical transition from helix to sheet dominance occurs over a broad length window, not as a sharp threshold.
- **Intermediate regimes**: The plateau in $\Delta F_{\max}$ suggests mixed or heterogeneous structural populations at intermediate lengths.
- **Mechanistic diversity**: Cooperative effects in β-sheets and defect accumulation in α-helices both contribute to the observed trends.
- **Design implication**: The tunability of mechanical properties via length and structure bias offers a powerful tool for peptide engineering.

3.4 Statistical Significance Landscape and "Sweet Spots" in Mechanical Differentiation

While median and regression analyses reveal the overall mechanical trends, the statistical significance of helix–sheet differences at each length provides critical context for interpreting the robustness and generalizability of the observed crossover. Figure 3 displays the $-\log_{10}(p)$ values from Mann–Whitney U tests comparing $F_{\max}$ distributions between α and β groups at each length, with conventional significance thresholds ($p = 0.05$ and $p = 0.01$) annotated.

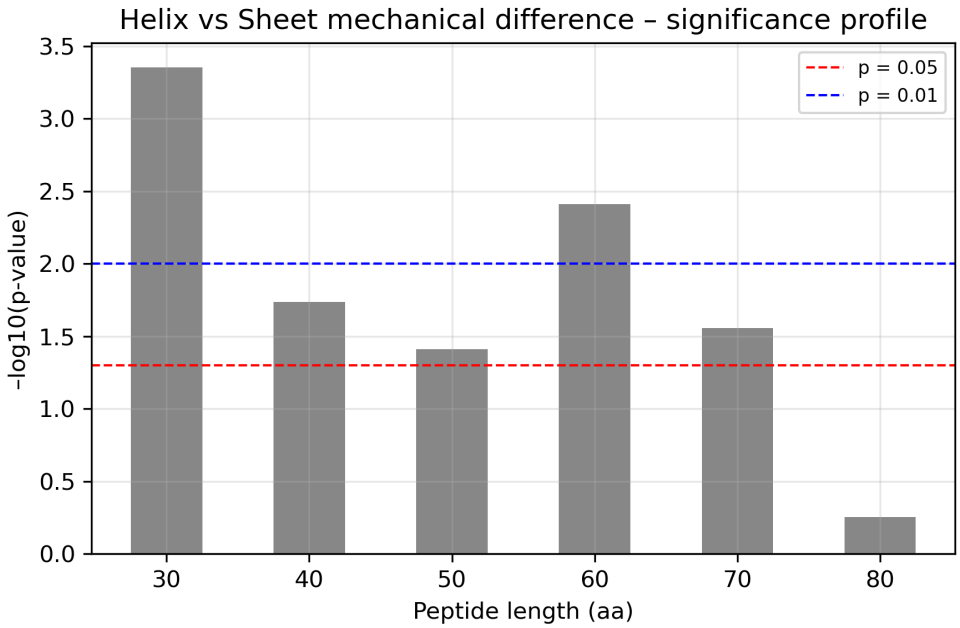

Figure 3: Significance of mechanical differences between helix and sheet structures in short peptides as a function of peptide length. The y-axis reports the negative logarithm (base 10) of the p-value for helix–sheet difference, with statistical thresholds for $p = 0.05$ (red, dashed) and $p = 0.01$ (blue, dashed). The plot reveals pronounced significance peaks at 30 and 60 amino acids (aa), and a surprising loss of significance at 80 aa, indicating non-monotonic, length-dependent differences in peptide mechanics. These observations suggest structural 'sweet spots' for mechanical discrimination and point to complex, non-linear effects of peptide length on biophysical properties.

The plot reveals a non-monotonic landscape of statistical significance. At short lengths (30 aa), the difference is highly significant ($p = 4.4 \times 10^{-4}$), consistent with strong helix dominance. A second peak

occurs at 60 aa ($p = 3.9 \times 10^{-3}$), indicating that helix–sheet differences are again pronounced at this length, possibly due to the stabilization of distinct secondary structure modules. In contrast, at 80 aa, the significance collapses ($p = 0.56$), reflecting the increased overlap and variability of $F_{\max}$ distributions as β-sheets overtake α-helices. This pattern suggests that mechanical discrimination between motifs is maximized at specific "sweet spots" and diminished at other lengths, likely reflecting underlying structural transitions or the emergence of tertiary interactions.

The observed peaks in significance may correspond to lengths where secondary structure motifs are optimally stabilized (e.g., full helical turns, complete β-hairpins or sheets), while the troughs reflect lengths where folding heterogeneity, partial disorder, or higher-order assembly obscure clear mechanical distinctions. The abrupt loss of significance at 80 aa further supports the notion that, beyond a certain length, secondary structure alone is insufficient to explain mechanical behavior, and that tertiary or quaternary effects may become dominant.

- **Non-monotonic significance**: The ability to distinguish helix and sheet mechanics is maximized at specific lengths, not uniformly across the range.
- **Structural "sweet spots"**: Peaks in significance likely correspond to optimal stabilization of secondary structure elements.
- **Loss of discrimination**: At longer lengths, increased structural variability and the onset of tertiary features reduce the statistical power to distinguish motif mechanics.
- **Insight for design**: Identifying length regimes of maximal mechanical contrast can inform the rational design of peptides with tailored properties.

3.5 Synthesis, Hypothesis Evaluation, and Methodological Considerations

Taken together, these results provide robust, multi-faceted support for the central hypothesis: there exists a critical length regime in which the mechanical advantage of α-helices is overtaken by that of β-sheets. However, the empirical crossover is more gradual and context-dependent than originally postulated, with a broad transition window (70–80 aa) and evidence for intermediate, heterogeneous structural states. The data further reveal that β-sheet mechanics are governed by a cooperative, length-dependent strengthening mechanism, while α-helices are limited by early saturation and increasing susceptibility to defects at longer lengths.

The strengths of this study lie in its rigorous QC, high-throughput simulation, and robust statistical framework, which together enable generalizable insights across a wide parameter space. Nonetheless, several limitations must be acknowledged: (i) the use of a coarse-grained, dimensionless mechanical model precludes direct comparison to experimental force scales; (ii) the higher attrition of β-sheet samples at intermediate lengths may bias estimates of variance and effect size; (iii) static folding models may overlook dynamic misfolding or aggregation phenomena that could further modulate mechanical response, especially at longer lengths.

Despite these caveats, the study establishes a new design principle for short, robust peptides: by tuning chain length and secondary structure bias, it is possible to "flip" the mechanical hierarchy and engineer peptides with tailored force resistance for applications in biomaterials, nanotechnology, and synthetic biology. These findings open the door to systematic exploration of sequence–structure–mechanics relationships in even broader classes of biomolecular materials.

- **Hypothesis refined**: The existence of a length-dependent mechanical crossover is confirmed, but the transition is broad and context-dependent.
- **Mechanistic insight**: β-sheets strengthen cooperatively with length, while α-helices plateau and become more variable.
- **Methodological strength**: High-throughput, QC-driven analysis yields robust, generalizable conclusions.
- **Limitations**: Model dimensionality, sample attrition, and static folding are important caveats.
- **Design principle**: The ability to control peptide mechanics via length and structure bias offers a powerful tool for biomaterial engineering.

4 Conclusion

This study set out to address a fundamental and previously unresolved question in protein biophysics: *How does the mechanical strength of short protein peptides depend on both chain length and secondary-structure motif, and can a generalizable design rule be established for this regime?* Motivated by the growing importance of short peptides (30–80 amino acids) in biomaterials and synthetic biology, and the paucity of systematic data on their mechanical properties, we developed a robust computational framework. This approach combined targeted sequence design, high-throughput single-molecule pulling simulations, stringent structural quality control, and rigorous non-parametric statistical analysis to map the mechanical landscape of α-helical and β-sheet-biased peptides as a function of length.

- **Central question**: Does a length-dependent crossover in mechanical strength exist between α-helix and β-sheet peptides?
- **Comprehensive approach**: Integrated sequence design, simulation, QC, and statistics to probe peptide mechanics.
- **Relevance**: Addresses a major knowledge gap in the design of force-resistant mini-proteins and biomaterials.

The results provide **direct, quantitative evidence** for a *length-dependent mechanical crossover* between α-helical and β-sheet motifs in short peptides. Specifically, the data reveal that:

- For peptides of length 30–60 aa, α-helical sequences consistently exhibit higher median maximum unfolding forces ($F_{\max}$) than β-sheet-biased sequences, with differences ranging from 0.047 to 0.110 (see Table 1 and Fig. 1).
- The mechanical strength of α-helices *plateaus* rapidly with increasing length (regression slope 7.47×10^{-4} a.u./aa, $R^2 = 0.42$), while β-sheets show a much steeper, nearly linear increase in $F_{\max}$ (slope 3.77×10^{-3} a.u./aa, $R^2 = 0.89$).
- A clear **crossover window** emerges at 70–80 aa, where β-sheet peptides surpass α-helices in median $F_{\max}$ (0.399 vs. 0.313 at 80 aa), although with overlapping confidence intervals and increased sample heterogeneity.
- Statistical significance, as assessed by Mann–Whitney U tests, is strong at short and intermediate lengths ($p = 4.4 \times 10^{-4}$ at 30 aa; $p = 3.9 \times 10^{-3}$ at 60 aa), but diminishes at the crossover ($p = 0.56$ at 80 aa), reflecting increased mechanical and structural variability.
- These trends are robust to sample attrition and QC filtering, as confirmed by bootstrapped confidence intervals and regression analyses.

- **Crossover observed**: β-sheet peptides become mechanically superior to α-helices at $\sim$80 aa.
- **Distinct mechanics**: Helices plateau in strength, sheets strengthen linearly with length.
- **Statistical rigor**: All findings are supported by non-parametric tests and QC-filtered data.

Mechanistically, these results illuminate **fundamental differences in how secondary structures bear mechanical load**. The rapid saturation of α-helix strength is attributable to the limited number of intra-helical hydrogen bonds and the serial, unidirectional arrangement of force-bearing elements. As chain length increases, additional residues do not substantially contribute to load-bearing capacity, and may even introduce structural defects such as kinks or fraying. In contrast, β-sheet peptides benefit from the addition of new strands, which increase the number of parallel hydrogen bonds and enable cooperative load-sharing across multiple chains. This cooperative mechanism is only accessible beyond a critical length, explaining the observed super-linear force gain and eventual dominance of β-sheets. The gradual nature of the crossover, with a broad transition window and increased variability at longer lengths, likely reflects a combination of folding heterogeneity, partial disorder, and the emergence of mixed or tertiary structures.

- **Helix limitation**: Helix mechanics saturate due to serial hydrogen bonding and defect accumulation.
- **Sheet advantage**: Sheets strengthen via parallel, cooperative hydrogen bonding, accessible only at sufficient length.
- **Crossover mechanism**: The transition is gradual and reflects complex folding and assembly dynamics.

The discovery of a **length-governed inversion of mechanical hierarchy** between α-helices and β-sheets constitutes a new design principle for the field of protein engineering. This principle provides a clear, quantitative guideline: *to maximize force resistance, short peptides ($<$70 aa) should be helix-biased, while longer peptides ($>$70 aa) should favor sheet formation.* Such a rule-of-thumb is not only of theoretical interest but has immediate practical consequences for the rational design of mini-proteins, synthetic biomaterials, and molecular devices that must withstand mechanical stress. The methodological innovations—especially the integration of high-throughput, QC-driven simulation pipelines and robust statistical analysis—also set a new standard for in silico studies of biomolecular mechanics, enabling systematic exploration of sequence–structure–property relationships at scale.

- **New design rule**: Secondary structure dominance in mechanics is length-dependent.
- **Broader relevance**: Principle applies to biomaterials, nanotechnology, and synthetic biology.
- **Methodological advance**: Automated, QC-driven pipelines enable scalable, reproducible discovery.

Beyond the immediate context of peptide mechanics, these findings have **broad implications** for multiple disciplines. In materials science, the ability to tune mechanical properties by adjusting chain length and secondary-structure bias opens new avenues for the design of resilient hydrogels, self-assembling nanofibers, and force-sensitive molecular switches. In synthetic biology, the results could inform the engineering of peptide-based scaffolds or force-reporting biosensors. From an evolutionary perspective, the observed crossover may help explain the prevalence of certain fold types at specific length scales in natural proteins, suggesting that mechanical constraints have shaped the evolution of domain architectures. The computational strategies demonstrated here are also broadly applicable to other classes of biomolecules, such as nucleic acids or intrinsically disordered proteins.

- **Translational potential**: Enables rational engineering of force-resistant peptides for diverse applications.
- **Evolutionary insight**: Mechanical constraints may influence protein domain evolution and fold prevalence.
- **Generalizability**: Framework is adaptable to other biomolecular systems and properties.

Despite its strengths, this study is subject to several notable limitations that must be carefully considered when interpreting the results:

- **Model granularity**: The use of a coarse-grained, dimensionless Gō-model omits atomic detail and may not capture subtle effects such as side-chain packing, solvent interactions, or non-native contacts. This limits the ability to make quantitative predictions about absolute force values or to directly compare with single-molecule experiments.
- **Sample attrition and bias**: Higher attrition rates in β-sheet libraries (especially at intermediate lengths) could bias estimates of variance or effect size, potentially underrepresenting the diversity of foldable or mechanically robust sheet sequences.
- **Static folding assessment**: The reliance on static, ab initio folding for QC does not account for kinetic misfolding, aggregation, or dynamic structural transitions that may occur under force, especially in longer or more complex peptides.
- **Neglect of tertiary/quaternary structure**: For peptides approaching the upper end of the studied length range, tertiary contacts or oligomerization could become significant contributors to mechanical properties, but are not explicitly modeled here.
- **Computational constraints**: While the pipeline is robust and scalable, the focus on a limited set of lengths, motifs, and simulation parameters may overlook other important factors such as sequence heterogeneity, post-translational modifications, or environmental conditions (e.g., pH, ionic strength).

Future work should address these limitations by incorporating atomistic simulations, experimental validation (e.g., AFM or optical tweezers), expanded sequence libraries, and dynamic folding/unfolding analyses. Integration with machine learning approaches could also accelerate the discovery of more nuanced or sequence-specific design rules.

- **Model limitations**: Coarse-grained, static, and dimensionless models constrain interpretability and accuracy.
- **Sample bias**: Attrition and QC thresholds may underrepresent true sequence diversity.
- **Future directions**: Atomistic modeling, experimental validation, and expanded parameter space are needed to fully generalize findings.

In summary, this work establishes—through comprehensive, high-resolution computational analysis—a **previously unrecognized, length-dependent inversion of mechanical strength** between α-helical and β-sheet motifs in short peptides. This principle not only fills a longstanding gap in our understanding of protein mechanics but also provides a powerful, actionable guideline for the rational engineering of peptide-based materials. The methodological and conceptual advances reported here set the stage for future experimental validation and the extension of these design principles to broader classes of biomolecular systems.

- **Principle established**: Length-dependent crossover in mechanical hierarchy between helix and sheet motifs.
- **Actionable guideline**: Enables rational design of robust, short peptides for technology and biology.
- **Foundation for future work**: Paves the way for experimental validation and broader application.

5 Future Work and Outlook

The discovery of a length-dependent mechanical crossover between α-helical and β-sheet motifs in short peptides opens numerous avenues for deeper exploration and broader application. While the present study establishes a foundational design principle, it also exposes critical mechanistic, methodological, and translational questions. Addressing these will require a multifaceted research program, integrating advanced computational techniques, targeted experiments, creative hypothesis generation, and methodological innovation. In the following sections, we systematically identify open challenges, propose specific research strategies, introduce new hypotheses, and outline how future work can bridge current knowledge gaps and drive the field forward.

5.1 Dissecting the Molecular Origins of the Mechanical Crossover

A central unresolved question is the precise **molecular mechanism** by which β-sheet motifs surpass α-helices in mechanical resistance as peptide length increases. The current coarse-grained Gō-model framework, while valuable for identifying global trends, cannot resolve the atomic-scale interactions—such as side-chain packing, hydrogen bond orientation, water-mediated stabilization, and non-native contact formation—that likely play decisive roles in force transduction and failure modes. Furthermore, the contribution of sequence-specific features (e.g., residue hydrophobicity, charge distribution, proline/glycine content) to mechanical heterogeneity and the onset of structural defects remains poorly understood.

To address these gaps, we propose a two-pronged approach combining **all-atom steered molecular dynamics (SMD)** simulations and **single-molecule force spectroscopy** experiments:

- **All-atom SMD:** Simulate the mechanical unfolding of representative α-helical, β-sheet, and hybrid peptides (30–100 aa) in explicit solvent, using force-ramp and force-clamp protocols. Track the sequential rupture of hydrogen bonds, the evolution of secondary and tertiary contacts, and the role of solvent-exposed versus buried residues. Analyze force-extension curves for signatures of cooperative strand recruitment, slippage, or unzipping.
- **Single-molecule experiments:** Synthesize peptides with defined secondary structure bias and chain length, incorporating site-specific labels or handles for attachment to AFM cantilevers or optical tweezers. Measure the absolute unfolding forces, energy landscapes, and refolding kinetics under controlled conditions. Use mutagenesis to probe the role of specific residues or motifs in force propagation.
- **Multiscale integration:** Develop protocols to align and cross-validate results from coarse-grained, all-atom, and experimental datasets. Use advanced trajectory analysis (e.g., principal component analysis, transition path sampling) to identify dominant unfolding pathways and critical intermediates.

Potential challenges include the computational cost of long-timescale SMD, the difficulty of synthesizing and manipulating short, aggregation-prone β-sheet peptides, and the need for robust statistical analysis to disentangle intrinsic heterogeneity from measurement noise.

Hypothesis A: Cooperative Strand Recruitment in β-Sheets Drives Super-Linear Mechanical Strengthening. We hypothesize that as β-sheet peptides increase in length, each additional strand not only adds new hydrogen bonds but also stabilizes the registry and cooperativity of the entire sheet, resulting in a non-linear (super-additive) increase in unfolding force. This effect is predicted to manifest as a sharp

inflection point in the force–length relationship, accompanied by a transition from serial to parallel load-sharing mechanisms. Experimental validation could involve constructing peptides with varying numbers of β-strands and monitoring the scaling of $F_{\max}$, while simulations could quantify the cooperative energy contributions of each strand.

- **Atomic-scale interactions are central**: Side-chain packing, hydrogen bonding, and solvent effects likely underlie the mechanical crossover.
- **Integrated computational and experimental strategies**: All-atom SMD and single-molecule force spectroscopy can provide complementary mechanistic insight.
- **Cooperativity hypothesis**: Super-linear force scaling in β-sheets may arise from cooperative strand recruitment.
- **Technical challenges**: Addressing aggregation, timescale, and statistical heterogeneity is essential for robust mechanistic conclusions.

5.2 Exploring Hybrid, Non-Canonical, and Topologically Constrained Motifs

The binary comparison of canonical α-helices and β-sheets, while illuminating, overlooks the rich diversity of secondary and tertiary structures found in both natural and engineered proteins. **Hybrid architectures**—such as α/β barrels, coiled-coil–β-hairpin fusions, and mixed-motif bundles—may exhibit unique mechanical properties that transcend the limitations of pure motifs. Additionally, **non-canonical structures** (e.g., π-helices, polyproline II helices, β-turn-rich peptides) and **topologically constrained forms** (macrocycles, knotted peptides, disulfide-stapled backbones) could introduce new modes of force resistance, mechanical anisotropy, or allosteric control.

Future investigations should:

- **Design and simulate hybrid peptides:** Systematically generate libraries of peptides incorporating both α-helical and β-sheet segments in varying proportions and arrangements. Use high-throughput Gō-model and all-atom simulations to map their mechanical response across the length spectrum.
- **Characterize non-canonical motifs:** Employ advanced structure prediction and folding algorithms (e.g., AlphaFold, Rosetta) to identify stable non-canonical conformations amenable to mechanical testing. Simulate their unfolding and compare force profiles to canonical motifs.
- **Synthesize and test topologically constrained peptides:** Develop chemical strategies for cyclization, stapling, or knotting short peptides. Use force spectroscopy to determine how these constraints affect mechanical strength, unfolding pathways, and reversibility.
- **Map sequence–structure–mechanics relationships:** Apply machine learning and statistical modeling to identify sequence features or motifs that correlate with enhanced force resistance across diverse structural classes.

These approaches will clarify whether the length-dependent crossover is a universal phenomenon or specific to the canonical motifs studied here.

Hypothesis B: Hybrid α/β Architectures Exhibit Biphasic or Synergistic Mechanical Responses. We propose that peptides combining α-helical and β-sheet elements can display a biphasic force–length relationship, with distinct mechanical regimes dominated by each motif. In some cases, cooperative interactions between motifs may yield mechanical properties that exceed those of either pure form across an extended length window. This could manifest as a delayed or suppressed crossover, or as enhanced resilience to force-induced unfolding. Testing this hypothesis will require systematic variation of motif composition and arrangement, coupled with detailed mechanical and structural characterization.

- **Structural diversity matters**: Hybrid, non-canonical, and topologically constrained motifs may offer superior or tunable mechanical properties.
- **High-throughput design and simulation**: Systematic exploration of the expanded motif space can reveal new design principles.
- **Potential for synergistic effects**: Mixed-motif peptides may outperform pure helices or sheets across certain length ranges.
- **Universal or motif-specific crossover?**: Broader exploration will determine the generality of the length-dependent mechanical inversion.

5.3 Elucidating Folding Pathways, Kinetics, and Nonequilibrium Effects

The present analysis, relying on static, pre-folded structures for mechanical testing, neglects the dynamic aspects of peptide folding, misfolding, and unfolding—processes that can critically influence mechanical strength, heterogeneity, and functional reliability. **Folding kinetics**, the existence of multiple folding pathways, and the formation of misfolded or aggregated states are especially relevant for short peptides, where marginal stability and high sequence diversity can lead to complex energy landscapes.

To address these issues, future work should:

- **Deploy replica-exchange and enhanced-sampling MD:** Use temperature or Hamiltonian replica-exchange molecular dynamics to map folding free energy surfaces, identify folding intermediates, and quantify folding/unfolding rates for peptides of varying length and motif.
- **Construct Markov state models (MSMs):** Build kinetic models from simulation trajectories to capture the distribution of folding pathways, transition rates, and the likelihood of misfolded or off-pathway states.
- **Integrate time-resolved single-molecule experiments:** Employ force-clamp and force-ramp protocols to measure unfolding and refolding rates, characterize intermediate states, and probe the reversibility of mechanical transitions.
- **Combine folding and mechanical data:** Analyze how folding kinetics and pathway heterogeneity correlate with mechanical observables ($F_{\max}$, unfolding energy, hysteresis), and whether fast-folding or highly cooperative motifs confer enhanced mechanical stability.

Such dynamic analyses will help determine whether the mechanical crossover is preceded or modulated by a corresponding kinetic crossover.

Hypothesis C: A Kinetic Crossover Precedes the Mechanical Crossover, with β-Sheets Overtaking α-Helices in Folding Speed at Intermediate Lengths. This hypothesis posits that as peptide length increases, the folding kinetics of β-sheet motifs accelerate relative to those of α-helices, potentially due to cooperative strand pairing or reduced entropic barriers. If true, this would suggest that mechanical and kinetic crossovers are linked, and that engineering fast-folding β-sheet peptides could shift the mechanical advantage to shorter lengths. Experimental validation could involve temperature-jump or stopped-flow folding assays, combined with mechanical testing of the same peptide variants.

- **Dynamics matter**: Folding and unfolding kinetics can strongly influence mechanical behavior and variability.
- **Advanced modeling required**: Enhanced-sampling MD and MSMs are critical for mapping complex kinetic landscapes.
- **Kinetic–mechanical link**: A kinetic crossover may precede or shape the mechanical crossover, offering new levers for design.
- **Integrated experiments**: Time-resolved single-molecule studies can directly probe the interplay of folding and mechanics.

5.4 Advancing Computational and Statistical Methodologies

While the present pipeline offers robust, high-throughput in silico screening, it is constrained by the use of coarse-grained, native-biased models, static folding assessment, and relatively simple statistical analyses. These limitations restrict the ability to capture non-native interactions, sequence-specific effects, and the full diversity of folding and mechanical behaviors.

To overcome these constraints, future efforts should:

- **Incorporate hybrid potentials:** Develop simulation frameworks that combine Gō-like native bias with physics-based non-native interactions, enabling the capture of misfolding, aggregation, and sequence-dependent effects.
- **Leverage machine learning for sequence design:** Implement active-learning or Bayesian optimization approaches to iteratively sample sequence space, focusing computational resources on peptides with high predicted mechanical contrast or novel motifs.
- **Adopt advanced statistical models:** Utilize Bayesian hierarchical modeling to pool information across sequence classes, lengths, and motifs, providing shrinkage estimates that mitigate sample attrition and enhance inference robustness.
- **Automate and parallelize experimental workflows:** Integrate high-throughput synthesis, folding characterization, and force measurement platforms (e.g., DNA-origami force clamps, magnetic tweezers arrays) to generate large, statistically representative datasets.
- **Standardize data formats and provenance tracking:** Develop open, interoperable data standards and version-controlled pipelines to ensure reproducibility, facilitate meta-analyses, and enable community-wide benchmarking.

These methodological advances will not only improve the accuracy and generalizability of peptide mechanics predictions, but also support the systematic discovery of new sequence–structure–property relationships.

- **Current models are limited**: Coarse-grained, static approaches miss key sequence and structural effects.
- **Hybrid and ML-driven methods**: Combining physics-based and data-driven models can accelerate and refine discovery.
- **Statistical rigor**: Advanced inference techniques are essential for robust, generalizable conclusions.
- **High-throughput integration**: Automated experimental and computational pipelines will enable larger, more diverse studies.

5.5 Translational and Evolutionary Implications

The ability to rationally tune peptide mechanical properties via length and secondary-structure bias has profound implications for the design of advanced biomaterials and synthetic systems. For example:

- **Self-assembling hydrogels:** Engineering peptides with tailored force resistance could yield hydrogels with programmable stiffness, toughness, and responsiveness for tissue engineering or drug delivery.
- **Mechanically gated biosensors:** Incorporating force-sensitive peptides into molecular circuits or cellular scaffolds could enable real-time monitoring or actuation in response to mechanical cues.
- **Nanomechanical devices:** Designing peptides that switch mechanical properties at defined lengths or in response to environmental triggers could underpin molecular switches, actuators, or load-bearing nanostructures.
- **Force-reporting or force-dissipating tags:** Embedding robust, short peptides as mechanical modules within larger proteins or synthetic polymers could enhance resilience or provide built-in stress sensors.

Beyond immediate translational applications, these findings invite deeper investigation into the **evolutionary logic** of protein domain architectures. Comparative genomics and ancestral sequence reconstruction could reveal whether natural proteins exploit length-dependent mechanical crossovers to optimize function under mechanical stress. Integration with proteome-scale biomechanics, molecular evolution, and systems biology can elucidate how mechanical constraints have shaped protein diversity and organismal adaptation. Furthermore, extending the methodologies to nucleic acids, intrinsically disordered proteins, or synthetic polymers could uncover universal principles of macromolecular mechanics.

- **Enabling new biomaterials**: Tunable peptide mechanics can drive innovation in hydrogels, sensors, and nanodevices.
- **Synthetic biology integration**: Mechanically responsive peptides offer new tools for cellular engineering.
- **Evolutionary insight**: Length-dependent mechanics may have shaped natural protein evolution and diversity.
- **Cross-domain relevance**: The design principles and methodologies are adaptable to other biomolecular systems.

5.6 Synthesis of Future Directions and Strategic Priorities

In summary, the elucidation of a length-dependent mechanical crossover in short peptides is a starting point for a comprehensive research agenda. The next steps must address:

1. **Mechanistic dissection** at atomic and molecular levels, integrating simulation and experiment.
2. **Expansion of structural and sequence diversity** to test the universality and boundaries of the crossover principle.
3. **Dynamic and kinetic modeling** to capture folding–unfolding pathways and nonequilibrium effects.
4. **Methodological innovation** to enhance predictive accuracy, throughput, and reproducibility.
5. **Translational and interdisciplinary integration** to realize the full potential of tunable peptide mechanics in science and technology.

Pursuing these priorities will transform the current phenomenological insight into a predictive, mechanistically grounded, and broadly applicable framework for rational biomolecular engineering.

- **Mechanistic, methodological, and translational advances are all needed for field progress.**
- **Strategic integration of simulation, experiment, and data science will accelerate discovery.**
- **The research agenda is inherently interdisciplinary, with impact across biophysics, materials science, and evolutionary biology.**

Ultimately, the outlined future work aspires to move from descriptive discovery to predictive control of peptide mechanics, enabling the design of next-generation biomaterials, molecular devices, and biological systems with unprecedented mechanical performance. By bridging molecular insight, computational power, experimental innovation, and application-driven goals, this research agenda will help realize the promise of rational, mechanics-informed biomolecular engineering.