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Characterization of Co-Existing Enfuvirtide Conformational States by Ion Mobility Mass Spectrometry and Hydrogen/Deuterium Exchange

OVERVIEW

PURPOSE: Assign and characterize multiple conformations in a therapeutic peptide sample

METHODS: Ion mobility mass spectrometry; solution-phase hydrogen/deuterium exchange LC-MS

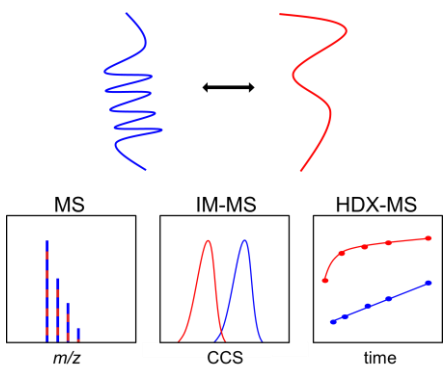
RESULTS: Multiple peptide conformations observed in both gas- and liquid-phases assigned as helical and disordered through the use of stabilized constructs

INTRODUCTION

Determining the purity of a simple peptide relies on identification and quantitation of structurally-related impurities. However, for more complex sequences which can exhibit higher-order structure, 'conformational purity' must also be considered [1]. This is of particular importance when a specific conformation represents the active form of the molecule. Ion mobility mass spectrometry (IM-MS) separates ions of identical *m/z* on the basis of collision cross section (CCS) in the gas phase. While proteins and their complexes can be transferred from solution into the gas phase without structural disruption, the fates of smaller peptides remain unclear. Both solution and instrument parameters can give rise to conformational artefacts [2], so experimentalists must remain vigilant to minimize such effects.

Hydrogen/Deuterium exchange (HDX) monitors conformational dynamics of the peptide backbone in solution, and the extent of exchange is driven largely by hydrogen bonding. Highly dynamic peptides often become maximally deuterated even at the earliest experimental time point that is manually feasible. Expanding the time window of exchange for monitoring dynamic systems can be accomplished by altering the temperature and/or pH [3].

In many cases peptide therapeutics are disordered in solution. This negatively affects their efficacy due to increased proteolysis and the entropic cost associated with folding into a bio-active state. Hydrocarbon-stapled peptide constructs often exhibit increased stability and activity [4]. Here we interrogate the conformational ensembles of a therapeutic peptide and a hydrocarbon-stapled variant.



Scheme 1. Conformer-specific outputs from MS-based techniques

METHODS

Materials

- Native Enfuvirtide

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2

- Stapled Enfuvirtide

Ac-YTSLIHSLIEESQNQQEKNEQELLEL**X**KWA**X**LWNWF-NH_2

Ion Mobility Mass Spectrometry

- Waters Synapt G2 QTOF
- TWIMS calibration with poly-D,L-alanine
- Gas-phase activation was minimized by voltage and temperature optimization
- Peptide solutions in 10 mM ammonium acetate, pH 5 were infused at 5 µL/min using a syringe pump
- IMS wave velocity: 800 m/s; wave height: 40 V

Hydrogen/Deuterium Exchange

- Isotope exchange by 15x dilution in D_2O-containing phosphate-citrate buffer at pH 5
- Reaction quenched at pH 2.5 and 0 ˚C
- Samples flash frozen in LN_2 and stored at -80 ˚C

LC-hrMS

- Samples manually injected onto switching valve in ice
- Rapid desalting and chromatography at 0 ˚C using Waters C8 column
- Mass analysis with Thermo Orbitrap Fusion Lumos
- Isotopic envelope analysis with HX-Express [4]

RESULTS

Ion Mobility of Native Enfuvirtide

Low Enf charge states exhibit multiple conformers in both positive and negative ionization modes

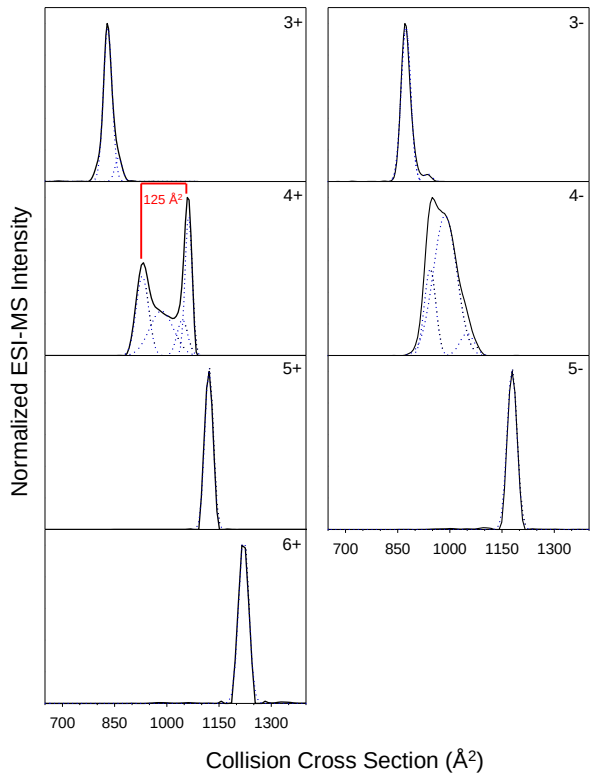


Figure 1. Native Enfuvirtide collision cross section distributions

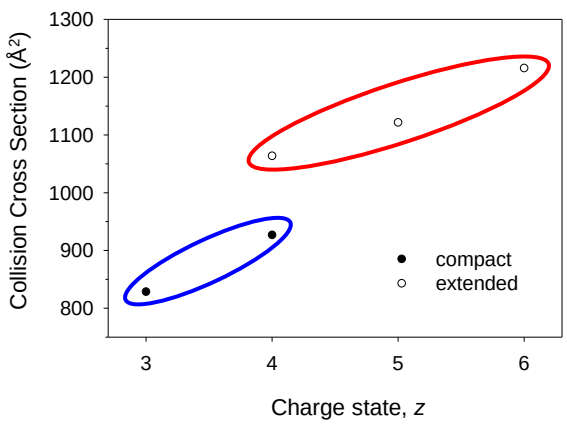


Figure 2. Native Enfuvirtide collision cross section distributions cluster into two conformer groups: compact and extended

Ion Mobility of Stablized Enfuvirtide

The *i*, *i+4* stapled Enfuvirtide construct cross-linked at residues 27 and 31 is highly helical in solution, as measured by CD

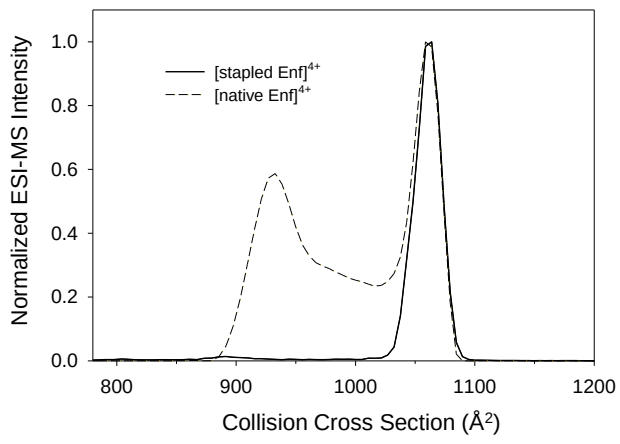


Figure 3. Hydrocarbon-stapled Enfuvirtide displays only a single conformer peak by IM-MS

Hydrogen Exchange Mass Spectrometry

HDX reports mainly on conformational dynamics by monitoring deuteration kinetics of peptide backbone amide hydrogens [5]

$$N-H_{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} N-H_{open} \xrightarrow[D_2O]{k_{ch}} N-D_{open} \underset{k_{op}}{\overset{k_{cl}}{\rightleftharpoons}} N-D_{closed}$$

$$k_{ch} \approx A e^{\frac{-E_a}{RT}} \cdot [OH^-]$$

Scheme 2. Simplified HDX mechanism. Intrinsic exchange rate scales with pH

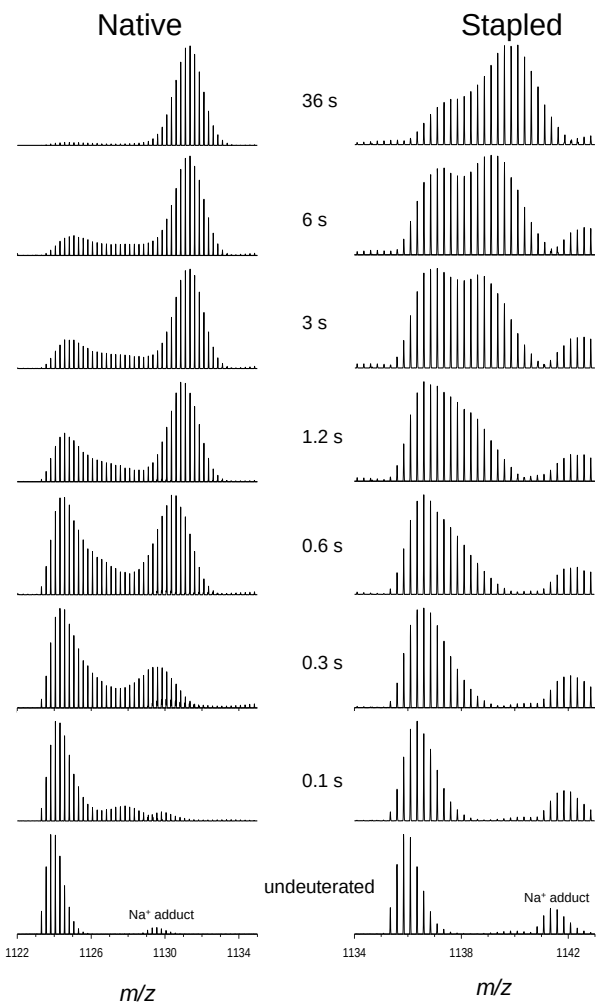


Figure 4. Multiple solution-phase Enf conformations revealed by HDX-MS. Exchange performed at pH 5, and reaction times converted to corresponding times at pH 7. The 4+ charge state is depicted for both peptides

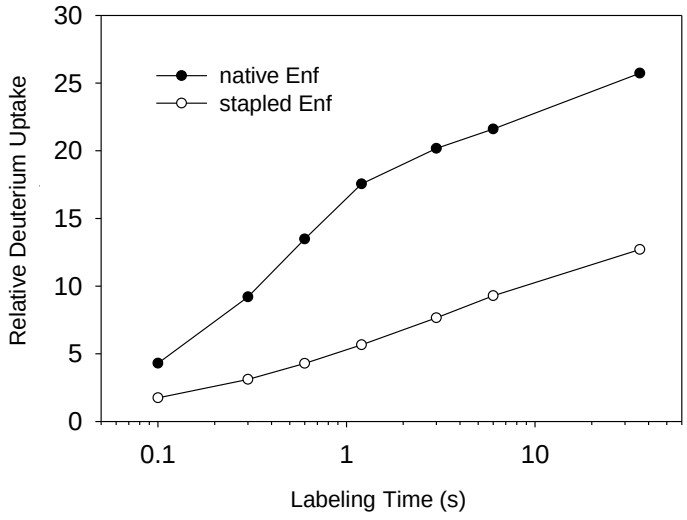


Figure 5. Deuterium uptake curves for native and stapled Enfuvirtide. Dramatic protection is observed for stapled construct, indicative of increased hydrogen bonding along the peptide backbone

CONCLUSION

IM-MS reveals multiple Enfuvirtide conformations in the gas-phase and the use of stabilized constructs allows for conformer assignment. Multiple populations are observed in solution by HDX for both native, and stapled Enfuvirtide, however the rate of deuterium uptake is dramatically reduced in the stabilized construct. Combination of IM-MS and HDX results gives insight into the conformational ensemble populated by a complex peptide, and this study provides a framework for conformational purity assignment in peptide reference materials.

REFERENCES

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